The Role of the Mitochondrial Permeability Transition Pore in Cardiac Myocyte Cell Death

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Summary

Ischaemia-reperfusion injury, sustained when the blood and oxygen supply is removed and then reinstated, is a common cause of cardiac tissue damage and therefore a popular area of scientific research.

It is commonly thought that the mitochondria play an important role in mediating ischaemia-reperfusion injury. Particular attention has been focused on the mitochondrial permeability transition pore. This has many times been shown to open only during the reperfusion phase disrupting the mitochondrial transmembrane potential and therefore the synthesis of ATP.

This study endeavours to examine the activity of the pore in rat neonatal primary cardiomyocytes during cell death. Models of apoptotic and necrotic cell death are established, as both have been implicated in ischaemia-reperfusion injury. An *in vitro* model of ischaemia-reperfusion injury is set up and an *in vivo* model of ischaemia-reperfusion injury is also examined.

Many studies have shown that inhibition of cyclophilin D can successfully block the opening of the mitochondrial pore. In this study, the cyclophilin inhibitor cyclosporin A is applied to determine the importance of cyclophilin D as an attenuator of ischaemia-reperfusion injury. The resulting effect on cell viability is analysed by various assays. Changes in the activity of the mitochondrial permeability transition pore are also monitored using a fluorescent mitochondrial transmembrane potential-sensitive dye.

A kinetic assay is established to measure the peptidyl prolyl *cis-trans*-isomerase activity of cyclophilin D, which is believed to have a role in regulation of the pore. Additionally ATP synthesis is measured by a luciferase-based assay and free radical production is monitored using a radical-sensitive dye during cell death. The effect of cyclosporin A on all measured parameters is examined.

The results presented in this study show that the importance of the mitochondrial permeability transition pore can vary widely according to the prevailing conditions. However, the data obtained in both isolated cardiomyocytes and cardiac tissue broadly uphold the concept of the major impact of mitochondrial permeability transition pore induction during ischaemia-reperfusion injury and strongly support the hypothesis of a significant role for cyclophilin D in the molecular mechanism of the pore.

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List of abbreviations

ADP	-	adenosine diphosphate
ANT	-	adenine nucleotide translocator
ATP	-	adenosine 5' triphosphate
AU	-	arbitrary units
BA	-	bongkrekic acid
Ca^{2+}	-	calcium ion
CAT	-	carboxyatracyloside
CCCP	-	carbonyl cyanide m-chlorophenylhydrazone
CsA	-	cyclosporin A
CyP	-	cyclophilin
CyPD	-	cyclophilin D
($DiOC_6(3)$)	-	3,3'dehexylocarbocyanine iodide
DMSO	-	dimethyl sulfoxide
ELISA	-	enzyme linked immunoassay
EM	-	electron microscopy
FADH	-	reduced flavin adenine dinucleotide
FCCP	-	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
FKBP	-	FK506 binding protein
H^+	-	hydrogen ion
H_2DCFDA	-	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	-	hydrogen peroxide
hr	-	hour(s)
g	-	gram(s)
l	-	litre
LAD	-	left anterior descending (branch of left coronary artery)
LDH	-	lactate dehydrogense
µl	-	microlitre
µM	-	micromolar
$m\Delta\Psi$	-	mitochondrial transmembrane potential
M	-	molar
MFI	-	mean fluorescence intensity
min	-	minute(s)
mM	-	millimolar
MPT	-	mitochondrial permeability transition
MPTP	-	mitochondrial permeability transition pore
MTT	-	3-(4, 5-dimethylthiazol-2-yl)-2-diphenyl tretrazolium bromide
mV	-	millivolts
NADH	-	reduced nicotinamide adenine dinucleotide
nM	-	nanomolar
p	-	probability
pH	-	potential of hydrogen (acidity)
Pi	-	inorganic phosphate
PI	-	propidium iodide
pO_2	-	oxygen partial pressure
PPI-ase	-	peptidyl prolyl *cis-trans*-isomerase
SOD	-	superoxide dismutase
SSP	-	staurosporine

t-BOOH	-	t-butyl hydroperoxide
TPP^+	-	tetraphenylphosphonium
TUNEL	-	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
VDAC	-	voltage dependent anionic channel
v/v	-	volume/volume
v.	-	versus
w/v	-	weight/volume
x g	-	times gravity

1: Introduction

1.1 Ischaemia-reperfusion injury in the heart – the clinical problem

Ischaemia-reperfusion injury is a well-established clinical problem sustained when the supply of blood and oxygen to the tissue is suspended (ischaemia) and then reinstated (reperfusion). It occurs in situations such as myocardial infarction, open-heart surgery and organ transplantation and is therefore a common cause of damage to solid tissues and a popular area of scientific research.

Restoration of the oxygen supply would logically be expected to limit tissue damage but it has long been recognised that major tissue damage can occur during the initial reperfusion phase (1-4). This may present a major problem following situations such as heart surgery or drug-induced thrombolysis after a myocardial infarction, as it could potentially result in patient death despite the procedure being otherwise successful.

1.2 Cell death during ischaemia-reperfusion injury – apoptosis or necrosis?

The two widely recognised forms of cell death are apoptosis and necrosis. Kerr, Wyllie and Currie first described apoptosis, also known as programmed cell death, as a physiological process in 1972 (5). Since then it has also gained recognition as a factor in many pathological situations, fuelling a vast amount of research into apoptotic inducers and signalling pathways.

Two types of signalling pathway have been identified for apoptosis – the 'extrinsic' and 'intrinsic' pathways. Extrinsic signalling pathways involve the activation of what are termed 'death receptors' on the surface of the cell and allow the cell to respond to its external environment (illustrated in Figure 1). These receptors, a subset of the tumour necrosis factor (TNF) receptor family, include TNFR1, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 and 2 and the Fas receptor. These are activated by binding of the appropriate ligand, which induces receptor trimerization and the recruitment and binding of other proteins to the intracellular 'death domain' (DD) possessed by these receptors. This results in the formation of a large complex known as the 'death-inducing signalling complex' (DISC) and the recruitment, oligomerization and activation of members 8 and 10 of the aspartic acid specific cysteine protease family (reviewed in 6), commonly known as caspases and the most important effectors of apoptosis discovered to date.

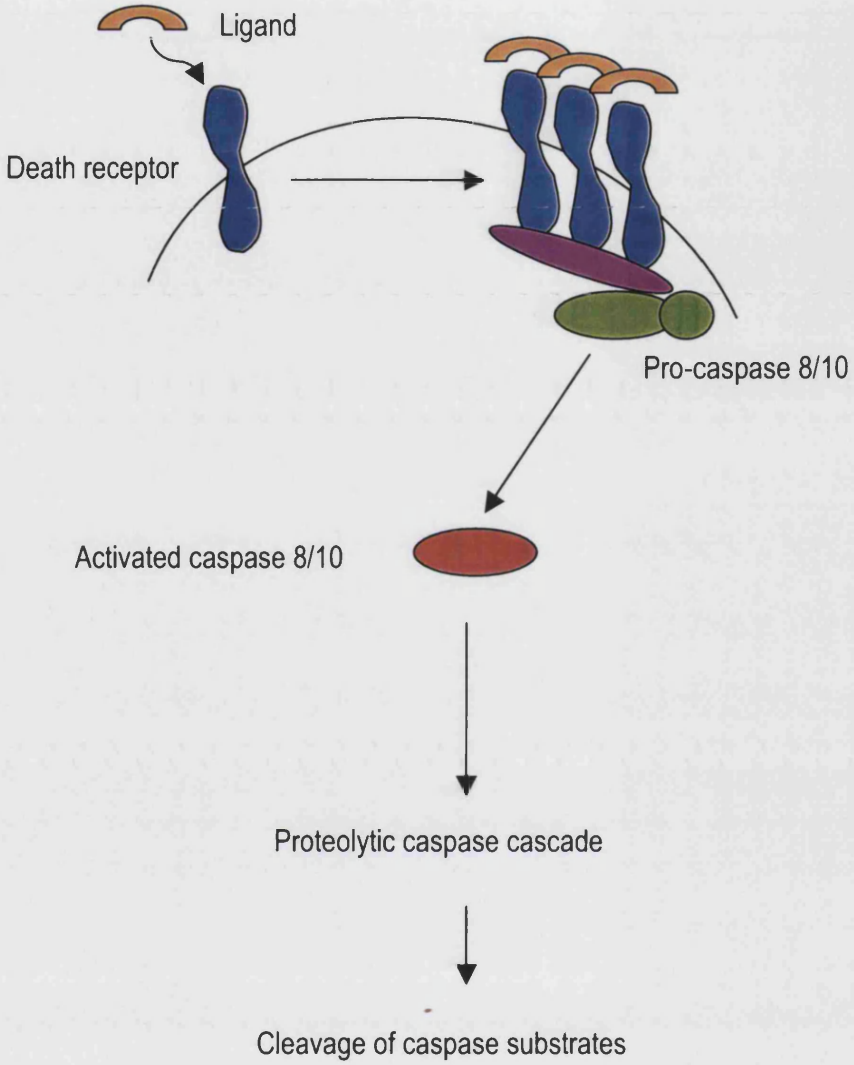


Figure 1: Extrinsic signalling through death receptors leads to activation of the apoptotic pathway. Binding of ligands to death receptors in the plasma membrane initiates a sequence of events culminating in morphological restructuring of the cell by activated caspase substrates. Thus the cell is able to response to unfavourable changes in the external environment.

The intrinsic signalling pathway occurs in response to an unfavourable intracellular environment and is most usually initiated from the mitochondria by the release of cytochrome c from the intermembrane space (illustrated in Figure 2). This may happen through the opening of a pore in the mitochondrial membranes termed the mitochondrial permeability transition pore (MPTP). Release of cytochrome c has frequently been demonstrated in conjunction with the opening of this pore (7-9), but it has also been demonstrated that apoptosis can proceed without any decline in mitochondrial membrane potential that is associated with induction of the pore (10-13), so the significance of cytochrome c release from the MPTP is unclear. An alternative explanation is that other proteins exhibiting pore-like activity, such as members of the Bcl-2 family, are responsible for regulating the release of cytochrome c. In particular, Bax and Bak are known to have a strong pro-apoptotic action, most probably through oligomerization to form pores in the outer mitochondrial membrane (14, 15), and possibly in conjunction with some elements of the MPTP (16, 17). It is known that other family members such as Bcl-2 and Bcl-xL are cytoprotective (14, 15, 18), but the exact mechanisms are yet to be elucidated. Upon release into the cytosol, cytochrome c binds to apoptosis-activating factor (Apaf)-1. In the presence of dATP these form a complex that recruits and activates caspase 9 (19) that then dissociates from the complex in order to cleave caspase 3 (20).

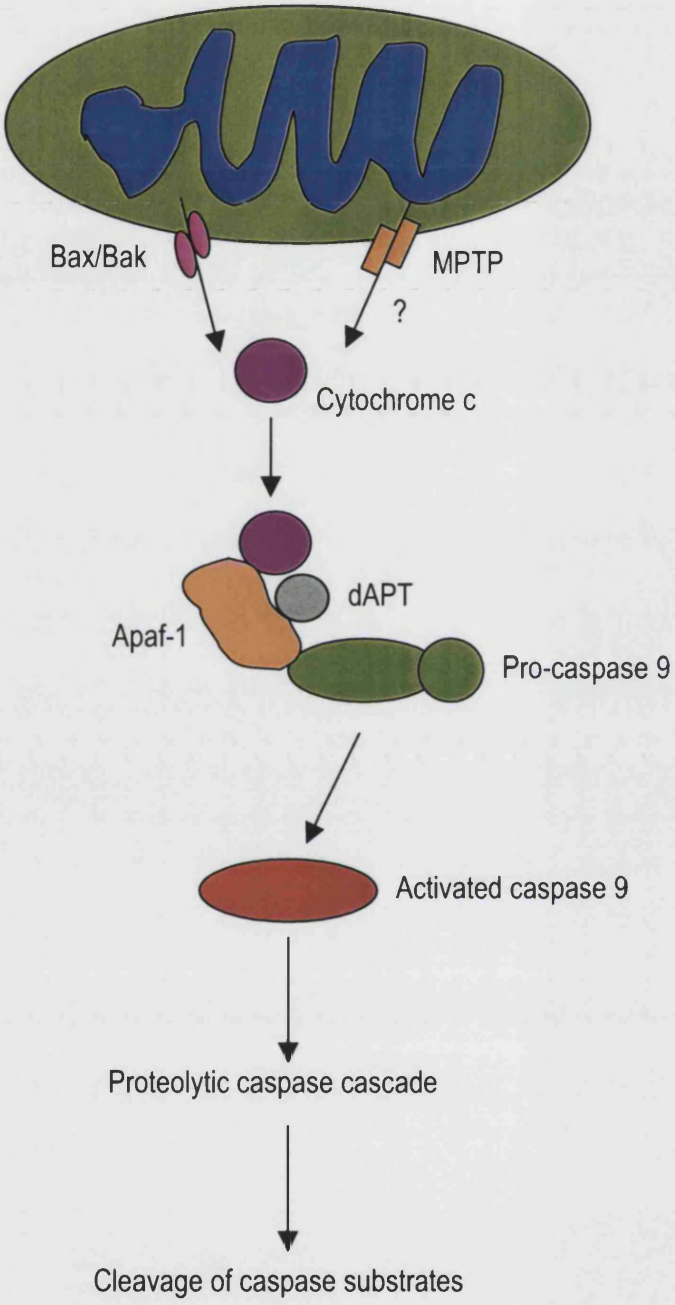


Figure 2: Intrinsic signalling, usually through the mitochondria, activates the apoptotic pathway. Release of cytochrome c through Bax/Bak and possibly through the mitochondrial permeability transition pore (MPTP) initiates a sequence of events culminating in morphological restructuring of the cell by activated caspase substrates.

Fourteen mammalian forms of caspases, the major executioners of apoptosis have so far been identified (reviewed in 21, 22). Those whose role in apoptosis is known fall into 2 groups. Apoptosis initiators, comprising caspases 2, 8, 9 and 10, contain either a caspase recruitment domain (CARD) and respond to activation of extrinsic pathways or a death effector domain (DED) and respond to initiation of the intrinsic signalling pathway. Both types recruit, cleave and activate the apoptosis effector caspases, comprising caspases 3, 6 and 7. Activated effector caspases can in turn cleave initiator caspases, creating a positive feedback loop. Besides this, other caspases are activated in a highly ordered proteolytic cascade (23), and other caspase substrates are cleaved in order both to implement the process of apoptosis and to prevent its inhibition.

Thus caspases activate gelsolin that, in turn, cleaves actin (24), activate DNA fragmentation factor that degrades DNA (25) and directly cleave structural proteins such as fodrin (26), vimentin and nuclear lamins A and B (27). A number of kinases are also cleaved, although the effects of these are less clear excepting a few specific cases such as the activation of MEKK-1 that can affect the rate of apoptosis through regulation of JNK signalling cascades (28, 29). To overcome inhibition of apoptosis, inhibitors of apoptosis proteins (IAPs) that regulate caspase activity are cleaved and inactivated to allow uninhibited caspase function (27), along with the DNA repair associated poly-ADP ribose polymerase (PARP) (30) in order to allow DNA fragmentation.

Although caspases are generally regarded to be the most significant effectors of the apoptotic process, some other proteins have been identified that may have a significant role. Calpains for example, have some substrates in common with the caspases and may be involved in the intrinsic signalling pathway (reviewed in 31). In addition, a number of regulators and inhibitors of apoptosis have been identified including p53 (reviewed in 32) and various kinases such as

phosphatidylinositol 3'-kinase (PI3K), mitogen-activated protein kinases (MAPK) and protein kinase A and C (reviewed in 33).

Ultimately, the substrates of caspase and other effectors restructure the dying cell in a well-known and clearly defined sequence of events that include chromatin condensation, nuclear DNA fragmentation, cell shrinkage, the dismantling of cell organelles and the formation of 'apoptotic bodies'. Neighbouring cells or cells of the immune system then engulf these bodies, thus avoiding spillage of enzymes and a consequential inflammatory response.

In contrast, necrosis is an uncontrolled event. One of the earliest visible signs of necrosis is the swelling and disruption of the mitochondria, as opposed to apoptosis where the mitochondria typically remain structurally normal. Necrosis does not proceed by any defined series of events and invariably results in the integrity of the plasma membrane being compromised, allowing the release of damaging enzymes into the extracellular environment.

The issue of the occurrence of apoptosis and necrosis in cardiac ischaemia-reperfusion injury has been the subject of much debate. The suggestion that apoptosis or a variation of the pathway may have a role in ischaemia-reperfusion injury has excited much interest in the process of cell death in this situation. Since the classically described process of apoptosis proceeds by a distinct and controlled pathway, it offers many more opportunities for intervention and inhibition than necrosis. This has led to the hope that the tissue injury occurring during ischaemia and reperfusion may potentially be prevented or at least substantially reduced through the inhibition of the apoptotic process.

The classical morphological symptoms of apoptosis have been described many times in cardiac cells (34-37). In addition, many of the biochemical markers of the apoptotic process, such as involvement of caspases (37-40), release of cytochrome c from the mitochondria (40-

42) and the appearance of DNA fragmentation (34, 43, 44) have been reported. Apoptosis has also been identified as a significant factor in a wide variety of cardiac disease states including idiopathic dilated cardiomyopathy (34, 45), acute myocardial infarction (46, 47), hypertension (48-50) and intractable congestive heart failure (51).

In the case of ischaemic injury, apoptosis has been reported in simulated *in vitro* ischaemia-reperfusion and isolated perfused organs (52), *in vivo* animal studies (44, 53, 54) and also in post-mortem human hearts (34, 45, 47). Conversely, there are also groups who have shown only the involvement of necrosis or an extremely insignificant occurrence of apoptosis in these very same situations (37, 55-57).

The main controversy surrounding this issue is the methods used to detect apoptosis. One of the most frequently employed methods is TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) staining, a technique relying on histochemical detection of the double-strand DNA breaks that are an accepted hallmark of apoptosis. However, it has been shown that TUNEL staining can also detect single-strand DNA breaks, which may occur during apoptosis or necrosis and also viable cells undergoing DNA repair (58). Additionally there are an increasing number of reports of cells displaying characteristics of both apoptosis and necrosis, such as histologically apoptotic cells with no DNA fragmentation (59) and cells that can be rescued from apparent necrosis by caspase inhibition (58). While this may be due to errors in detecting and defining apoptosis, it is also becoming increasingly likely that apoptosis and necrosis are simply two extreme examples of the process of cell death. The actual pathway by which the cell dies may actually be a combination of both recognised processes, with factors including the availability of ATP energy determining precisely how cell death will be executed. A defining characteristic of studies in these areas is that severe ischaemic conditions result in necrotic cell death whereas milder forms of ischaemia tend to induce apoptosis. This is supported by more recent studies demonstrating that the main area

of cardiac infarct is subjected mostly to necrotic cell death whereas myocytes on the edge of the infarct area and those somewhat distant from the area of main damage die through the slower process of apoptosis (53, 60). It is also now considered that apoptosis may be a vital element of the cardiac remodelling that can occur subsequent to ischaemic damage (53).

Several studies have now demonstrated that the availability of ATP may be a crucial factor determining whether apoptosis can be implemented or whether the cell will succumb to necrosis (61-63). As the majority of ATP is generated by the mitochondria located respiratory chain, this immediately suggests an important role for the mitochondria in cell death.

1.3 The mitochondria

1.3.1 Structure and function of the mitochondria

The mitochondria are frequently described as the powerhouse of the cells, as they produce the vast majority of the energy required to drive essential intracellular reactions via the production of ATP. The outer mitochondrial membrane that encloses the mitochondria is non-selectively semi-permeable, allowing all molecules of 5000 Daltons or less to readily pass into the intermembrane space. The highly specialised inner mitochondrial membrane is highly convoluted and forms a series of inward projections known as cristae. This membrane is virtually impermeable but contains transport proteins that selectively regulate the passage of specific metabolites and inorganic ions in and out of the matrix space that it encloses. Also contained in the inner mitochondrial membrane is ATP synthase and the proteins that carry out the reactions of the respiratory chain. The cristae provide a greater surface area for the containment of these various proteins, and mitochondria taken from cardiomyocytes contain more cristae than mitochondria from other sources due to the greater requirement for ATP in the constantly contracting cardiac myocytes.

A small proportion of ATP is produced in the cytosol by glycolysis, but pyruvate generated from this process is subsequently actively transported into the mitochondrial matrix where the tricarboxylic acid (TCA) cycle occurs. The high-energy electrons that result from this process are then passed along the inner mitochondrial membrane-bound components of the respiratory chain generating the electrochemical gradient. This in turn drives the process of oxidative phosphorylation that produces the majority of ATP.

1.3.2 The importance of ATP

ATP consists of adenosine, ribose and 3 phosphate groups. The terminal phosphate groups are highly reactive so that their hydrolysis or transfer to another molecule is an extremely

thermodynamically favourable reaction. Specialised enzymes can then couple this reaction to other, energetically unfavourable cellular reactions. For this reason ATP is the principle carrier of chemical energy in the cell. Reactions that are ATP-dependent include protein synthesis, active transport across membranes and muscle contraction.

1.3.3 The respiratory chain

The process of the TCA cycle based in the mitochondrial matrix results in the production of high-energy electrons contained in $FADH_2$ and NADH. $FADH_2$ is membrane bound and feeds electrons directly into one of the protein complexes of the respiratory chain, whereas NADH passes electrons to the components of the respiratory chain via random collisions with the membrane-bound protein carriers.

There are a large number of membrane-bound electron carriers in the respiratory chain. These are associated into 4 complexes - NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), ubiquinol-cytochrome c reductase (complex III) and cytochrome oxidase (complex IV), with coenzyme Q and cytochrome c acting as electron carriers between the complexes. Electrons are passed along the respiratory chain through electron carriers with increasingly greater redox potentials (affinity for electrons) until they are eventually passed to oxygen and combined with $2H^+$ to form H_2O.

The transfer of electrons between the complexes of the respiratory chain is an energetically favourable reaction that is coupled to the unfavourable reactions required to pump protons out of the matrix into the intermembrane space. This movement of protons creates an electrical and pH gradient due to the higher concentration of H^+ outside the inner mitochondrial membrane. This electrochemical gradient creates a proton-motive force that is measurable at approximately –180mV and is generally referred to as the mitochondrial transmembrane potential or $m\Delta\Psi$. Protons naturally flow down this gradient back into the matrix. They

penetrate the inner mitochondrial membrane by passing through ATP synthase, a large protein complex that utilises the energy to synthesize ATP from ADP and inorganic phosphate (Pi). The whole process of generating energy through the respiratory chain to drive ATP synthesis is known as oxidative phosphorylation and is essential for the viability of the cell.

1.3.4 Regulation of calcium

Ca^{2+} is a ubiquitous and crucially important intracellular signalling agent in all mammalian cells. Extracellular levels of Ca^{2+} are very high compared to the low cytosolic concentration, thus creating a large gradient across the cytosolic membrane. The activation of a Ca^{2+}-sensitive receptor causes entry of Ca^{2+} into the cytosol triggering Ca^{2+} responsive processes in the cell. In order for this mechanism to function successfully, Ca^{2+} must be continuously removed from the cytosol to maintain a low concentration. This is achieved largely through the action of Ca^{2+} transporters located in the membranes of the cell and the endoplasmic reticulum. These transporters pump Ca^{2+} across the membrane, utilising the energy of ATP hydrolysis so that Ca^{2+} can be moved against even a large gradient. In addition some cells including cardiomyocytes also contain an antiporter that can move Ca^{2+} out of the cell in exchange for Na^{+}. This mechanism however, has a relatively low affinity for Ca^{2+} and comes into effect only under conditions of greatly increased cytosolic Ca^{2+} concentration. A further mechanism for the removal of cytosolic Ca^{2+} is contained in the membrane of the mitochondria. This low-affinity, high capacity uniporter transports ions down its electrochemical gradient into the mitochondrial matrix but is dependent upon the mitochondrial transmembrane potential for its activity. It has been demonstrated that the uniporter cannot function in the absence of high $m\Delta\Psi$, even in the presence of a high Ca^{2+} gradient (64). Ca^{2+} is returned into the cytoplasm largely via the Na^{+}-Ca^{2+} ion exchanger except in exceptional circumstances as will be further discussed. There is now a substantial body of evidence that the mitochondria are almost invariably involved in

cellular Ca^{2+} signalling, notably in the control of cellular metabolism through upregulation of the TCA cycle. Although the majority of this work has been in non-cardiac cell types (65-67), there are also some publications demonstrating the ability of mitochondrial Ca^{2+} to influence cardiac cell metabolism (68-70) and various models of Ca^{2+}-dependent cardiomyocyte metabolism have now been proposed (71-73).

1.4 The Mitochondria in Ischaemia

1.4.1 Ischaemic conditions in the cell

As detailed above, the presence of oxygen in the cell is essential for the correct functioning of the respiratory chain. During ischaemia the lack of oxygen forces the cells to rely only on glycolysis for the production of ATP. This naturally results in the build-up of lactic acid that in turn inhibits glycolysis (74) exacerbating the depletion of adenosine nucleotides and the corresponding increase in inorganic phosphates and creating an acidic pH.

Intracellular pH is regulated by the active transport of ions across the plasma membrane, largely by ATP-dependent transporters and ion exchangers. H^+ is driven out of the cell in exchange for Na^+. In an ischaemic environment, this accumulation of Na^+ combined with the effects of oxidative stress (75), render the Na^+-Ca^{2+} ion exchanger unable to remove Ca^{2+} from the cytosol (76, 77). In addition, the depleted levels of ATP impair the activity of the ATP-dependent Ca^{2+} transporter (78, 79) resulting in the accumulation of cytosolic Ca^{2+} associated with ischaemia (80, 81).

1.4.2 Inhibition of the mitochondrial respiratory chain and generation of superoxide

The effect of ischaemia on the respiratory chain has been studied in mitochondria isolated from a variety of animal models of ischaemic injury, including a number of cardiac studies. It has been found that ischaemia causes significant inhibition of complexes I (82-87), II (85, 87)and III (85), and also mitochondrial ATPase (complex V) (83). Low pH is one of the cited causes of respiratory chain enzyme inhibition and has been demonstrated for complex I (84). Complex IV however appears to be unaffected by ischaemia (85).

Inhibition of respiratory chain complexes inevitably leads to a fall in ATP production and impairment of ATP-dependent ion pumps as well as compromising other important ATP-dependent reactions.

Ischaemic inhibition results in the respiratory chain complexes becoming reduced (88, 89) as they harbour electrons instead of passing them along the chain to O_2 as normal. This results in the production of free radicals as the complexes of the respiratory chain randomly pass the excess electrons to residual oxygen creating superoxide radicals (90). A number of studies of simulated ischaemia in both isolated cells and whole tissue have shown that the major sites of superoxide production appear to be complexes I and III (91-94). This concurs with the finding that these complexes suffer the most severe inhibition during ischaemia (95-99).

An extremely important factor in the generation of superoxide by the respiratory chain is the concentration of residual oxygen in the cardiac tissue. It has in fact been shown that removal of oxygen prevents the generation of free radicals and therefore limits damage (100).

Another frequently cited source of free radicals in cardiomyocytes is xanthine oxidase. However, there is apparently extremely little detectable xanthine oxidase activity in human cardiac tissue so this system may not be an important generator of free radicals from a clinical point of view (101).

It has long since been established that various reactions in the cell produce free radicals under normal physiological conditions, including the respiratory chain. Therefore cells contain important defence mechanisms against free radicals. Superoxide radicals are generally degraded to produce H_2O_2 and O_2, a reaction that is catalysed by superoxide dismutase (SOD) or by spontaneous dismutation. In the mitochondria the manganese-associated form, MnSOD, performs this function. During ischaemia however, free radicals are generated at a higher than normal level and moreover, a number of studies have shown widespread inhibition of mitochondrial enzymes during ischaemia, including MnSOD (102-105). This results in the production of free radicals at a faster rate than can be effectively removed, leading to further suppression of oxidative phosphorylation (106).

1.5 The Mitochondria in Reperfusion

1.5.1 Generation of hydrogen peroxide and hydroxyl radicals

As discussed in the previous section, under ischaemic conditions the complexes of the respiratory chain are inhibited and become reduced resulting in the donation of electrons to residual oxygen to produce superoxide.

It was shown, as early as the 1970s, that superoxide is merely the precursor of more potent free radical products. Although deliberately generated superoxide has been shown to have extremely toxic effects on numerous occasions, hindsight has attributed this to the action of hydroxyl radicals (107). Many studies have since shown that scavengers of the various precursors of hydroxyl radicals can significantly attenuate ischaemia-reperfusion injury in a wide variety of situations. Although most studies have applied free radical scavengers continuously throughout the period of ischaemia as well as reperfusion (108-110), a study by Ambrosio and colleagues applied SOD only during reperfusion. This study in an open-chest dog model, found that application of SOD resulted in a significant reduction in infarct size (111). This highlights the significance of the burst of free radical generation observable upon reperfusion that is presumably due to the donation of electrons to the restored oxygen.

There are now reports also demonstrating significant generation of H_2O_2 and hydroxyl radicals following ischaemia-reperfusion injury that may be attributable to the degradation of superoxide radicals produced during ischaemia (90, 100). As the superoxide is present in greater amounts than normal, an excess of H_2O_2 is consequently produced. Under physiological conditions, H_2O_2 is degraded to safe, non-reactive products by peroxidases and catalases. However during ischaemia-reperfusion injury, free radicals are produced in a quantity that eclipses the activity of the endogenous antioxidant defences (92), an effect that is exacerbated by the aforementioned ischaemic suppression of mitochondrial enzymatic activity.

This combination of circumstances allows the natural breakdown of H_2O_2 to produce hydroxyl radicals. This reaction is especially likely to occur in the mitochondria due to the presence of iron-based compounds in the respiratory chain (112) that may encourage the reduction of H_2O_2 via the Fenton equation:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\bullet \text{ (hydroxyl radical)}$$

Hydroxyl radicals are extremely reactive and can cause widespread damage by attacking membranes, DNA and other essential cell components. Unlike superoxide and H_2O_2 no specific endogenous enzymes are produced to scavenge them since under physiological circumstances the generation of hydroxyl radicals is avoided by the catalytic conversion of H_2O_2 to non-reactive products.

1.5.2 Uptake of calcium

During ischaemia excess Ca^{2+} accumulates in the cytoplasm as detailed above. Upon reperfusion the depleted levels of ATP prevent Ca^{2+} being removed by ATP dependent ion transportation (113). Instead, the pathologically high levels of cytosolic Ca^{2+} cause activation of the Ca^{2+} uniporters, which take up Ca^{2+} into the mitochondrial matrix (80, 81, 114). Due to the multiple Ca^{2+} binding sites contained in the matrix, mitochondria are able to effectively buffer Ca^{2+} and accumulate large quantities before a rise in free matrix Ca^{2+} can be observed (80, 81). Eventually however, the rise in matrix Ca^{2+} is sufficient to cause efflux of Ca^{2+} from the mitochondria through the process of mitochondrial permeability transition.

1.5.3 Onset of mitochondrial permeability transition

During reperfusion, the sudden increase in oxygen combined with reduced respiratory chain complexes produces free radicals as described above. This ischaemic inhibition of the respiratory chain also results in depleted levels of ATP and a consequent accumulation of inorganic phosphates. All of these circumstances are known to contribute to the onset of mitochondrial permeability transition, induced by the high matrix Ca^{2+} concentration detailed above.

During mitochondrial permeability transition (MPT), the inner mitochondrial membrane that is normally selectively permeable only by active transport, becomes non-specifically permeable to molecules of < 1500 Daltons. As early as 1979, Hunter and Haworth first proposed the existence of a channel to account for the phenomenon of MPT (115, 116). This theory was subsequently lent much weight by the potent inhibition of MPT by cyclosporin A (CsA) described by Crompton and co-workers (117).

1.6 The mitochondrial permeability transition pore

This pore has since been termed the mitochondrial permeability transition pore (MPTP) and has come under intense study due to its reported influence on both necrotic and apoptotic cell death (118-122). It is held that during necrosis, the levels of ATP are too low to sustain cell functionality and the MPTP opens irreversibly releasing Ca^{2+} into the cytosol. The mitochondria become swollen and disrupted and functionally collapse leading to the death of the cell (123, 124). During this process the plasma membrane becomes compromised and damaging intracellular enzymes are released into the surrounding tissue potentially triggering an inflammatory response. Apoptosis however is an energy-requiring process, which can only operate in conditions of high ATP (61, 62). Reduction of the transmembrane potential has been demonstrated in many systems and this is considered by many to be due to the opening of the MPTP (125, 126). However, it is thought that this may be a transient, reversible opening since ATP synthesis is necessary for apoptosis to be fully implemented. Opening of the pore may allow the release of pro-apoptotic factors such as pro-caspase 3, apoptosis-inducing factor and cytochrome c from the intermembrane space. Alternatively this may occur via an alternative method, for example the proposed release of cytochrome c via Bax (15).

As mentioned previously, both apoptotic and necrotic cell death have been cited as major factors in ischaemia and reperfusion induced damage suggesting a role for the MPTP. Additionally, many studies have now produced clear evidence for the importance of the MPTP in reperfusion in its own right. Passage of molecules through the mitochondrial membranes consistent with the induction of the MPTP has frequently been observed upon reperfusion (127-130), demonstrating that MPTP is almost certainly a significant pathological event. Studies with CsA, which inhibits the MPTP, have served to confirm this hypothesis. CsA offers potent protection against simulated ischaemia in both isolated mitochondria (131, 132) and

cultured cells (132-134) and perhaps most tellingly, has also been shown to protect a variety of whole tissues (133, 135-138) from reperfusion injury, indicating that the process is not merely an *in vitro* artefact. In addition, various reports have shown that protection against reperfusion related tissue damage is only provided by analogues of CsA that block the MPTP in isolated mitochondria (139, 140). This gives strong evidence that it is the direct influence of CsA on the MPTP that is the crucial factor as opposed to other effects such as the ability of complexes of CsA and cyclophilin (CyP) to bind calcineurin.

The effect of MPTP induction has been studied experimentally in a variety of models of cell death. Many of the publications on this subject have taken the release of cytochrome c into the cytoplasm as evidence for the involvement of permeability transition in the cell death process (7, 141). Although release of cytochrome c has previously been accepted as proof of the opening of the MPTP, recent studies have shown that release may occur independently of a reduction in mitochondria transmembrane potential (10) and therefore be achieved without any disruption to mitochondrial function (11). However, many groups studying the effect of the MPTP have done so using more direct assays of mitochondrial viability. Measurement of the transmembrane potential has shown a reduction during apoptosis induced by cytotoxic T lymphocytes (142), and treatment of primary thymocytes with MPT inducers such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) and protoporphyrin IX produced some of the classical signs of apoptosis along with reduction of transmembrane potential (119). Furthermore, some groups have developed the methodology to directly measure the opening of the MPTP as opposed to merely measuring $m\Delta\Psi$. These assays have largely been based on the inability of certain substances to enter the mitochondria unless the pore has opened. Crompton and colleagues use of radiolabelled sucrose to track the permeability of the inner mitochondrial membrane produced some of the earliest convincing evidence for a non-specific

mitochondrial pore (127, 143, 144). Halestrap and co-workers used entry of [^{3}H]-DOG (deoxyglucose) into the mitochondria of isolated, perfused hearts to show that the MPTP opens only during the reperfusion phase of ischaemia-reperfusion injury (130). Additionally, Lemasters and colleagues have developed the use of the fluorochrome calcein as an assessment of pore opening during tumour necrosis factor-α and simulated ischaemia-reperfusion injury (145), although this protocol has proved to be somewhat controversial as will be further discussed (see Chapter 8.1.2). Using these direct methods, it has been shown that inhibitors of the MPTP can inhibit apoptosis *in vitro* (118, 145-149) and it has also been shown that CsA can protect isolated perfused hearts against ischaemia-reperfusion injury (124). Conversely, Minamikawa and colleagues showed that a human osteosarcoma cell line could undergo MPT reversibly without the occurrence of cell death (150).

1.6.1 Consequences of mitochondrial permeability transition pore induction

Therefore the importance of the MPTP in any form of cell death remains a much-debated point. However, the fact that mitochondria are the generators of the vast majority of cellular energy makes it extremely likely that preservation of the mitochondrial transmembrane potential is essential for the survival of the cell. The induction of a pore in the inner mitochondrial membrane has a potentially disastrous effect on oxidative phosphorylation. As the electrochemical gradient is maintained by the strictly controlled passage of ions across the membrane any major increase in permeability will result in a random influx of ions and consequent collapse of the gradient. This destroys the driving force behind ATP synthase so that no ATP can be generated via oxidative phosphorylation with predictable consequences for ATP-dependent reactions in the cell. Some of these reactions such as protein folding are particularly vital in a post-ischaemic environment where the cell is attempting to recover from injury. Lack of ATP may turn reversible damage into irreversible damage or it may result in a

potentially apoptotic cell undergoing necrosis that could facilitate a damaging inflammatory response.

It has been suggested that the $m\Delta\Psi$ may fluctuate in a normal physiological state and that this may be accounted for by the MPTP opening transiently (127, 151). It is clear however that any prolonged opening of the pore would destroy the $m\Delta\Psi$ and therefore be disastrous for energy production in the cell.

1.7 Molecular structure of the mitochondrial permeability transition pore

The exact structure of the MPTP is not yet completely clear, as a number of groups have published studies containing potentially conflicting material. The one component of the pore that remains undisputed is the adenosine nucleotide transloctor (ANT), and cyclophilin D (CyPD) is widely accepted as a potentially essential element. Beyond that there are a large number of molecules including the voltage dependent anionic channel (VDAC), hexokinase, Bcl-2 and Bax that have been found in association with other elements of the MPTP under varying circumstances.

1.7.1 The adenosine nucleotide translocase

ANT is an abundant mitochondrial protein located in the inner membrane. Under physiological circumstances it mediates the exchange of ADP-ATP across the membrane, an essential process that supplies ATP to the cytosol. Hunter and Haworth first suggested involvement of the ANT in the MPTP in 1979 (115, 116) and a subsequent study by LeQuoc and LeQuoc (152) demonstrated the ability of reagents that stabilise the ANT in one of its 2 conformations, to markedly affect the sensitivity of the MPTP to [Ca^{2+}]. More recently, patch clamping of isolated ANT contained in liposomes demonstrated the ability of ANT to form a high conductance channel in the presence of Ca^{2+} (153) and another study of vesicle bound, reconstituted ANT showed Ca^{2+}-dependent release of small molecules (154).

1.7.2 The voltage dependent anion channel

The VDAC (also known as porin) is the most abundant protein in the outer mitochondrial membrane and as with the ANT, is involved in cross membrane transport under normal circumstances. It has been demonstrated that VDAC binds strongly to the inner mitochondrial membrane, creating contact points through association with ANT. Experiments with patch

clamped mitochondria membranes showed that a high conductance, CsA-sensitive channel displayed some characteristics associated with VDAC (155) and led to the possible scenario that the MPTP may naturally occur at these points of contact between the inner and outer mitochondrial membranes. This theory has been lent weight by recent evidence that the ANT-1 isoform, which is located exclusively in the peripheral inner mitochondrial membrane, has a far higher affinity for CyPD than the ANT-2 isoform, which is located both peripherally and in the cristae (156).

Some groups such as that of Halestrap dispute the importance of VDAC involvement in the MPTP. They have published studies demonstrating that the only components necessary to replicate the established behaviour of the MPTP are ANT and CyPD, suggesting that VDAC is likely to play only a regulatory role (157). However, the fact that so many studies have now successfully purified complexes of VDAC-ANT and also VDAC-ANT-CyPD has lent weight to the proposal that it may be an integral and important component of the MPTP *in vivo* (156, 158, 159). This of course does not rule out the option that ANT-CyPD complexes may form the MPTP alone especially as the purification protocols vary between the studies and in some cases the methodology is under dispute. However, although mitochondria contain approximately 10 times more ANT than VDAC, much of the excess ANT may be accounted for by the cristae located isoform so some if not all of the CyPD-binding isoform of ANT may also be complexed with VDAC.

One extremely interesting point to result from the study of VDAC-ANT complexes is the finding by Crompton and colleagues that VDAC-ANT-CyPD complexes will readily assemble in the absence of factors required for MPTP induction. The same group also showed application of ADP to isolated mitochondria increased the amount of free CyPD detectable in the matrix by radiolabelled, photoactive CsA (160, 161). These findings strongly suggested that CyPD can be found associated with ANT in physiologically normal isolated mitochondria and led the

authors to propose that the MPTP complex may form and function under physiological conditions.

A number of other molecules have been found to be associated with VDAC-ANT complexes some of which have been shown to play a regulatory role. Complexes of VDAC-ANT reconstituted in vesicles were seen to display Ca^{2+} sensitive MPTP-like activity as judged by the release of malate from the vesicles. When octameric mitochondrial creatine kinase was associated with the complex through binding to ANT, MPTP-induction was completely inhibited. When cytosolic hexokinase was also associated with the complexes through binding to VDAC, it was found that MPTP-induction was inhibited or encouraged by, respectively, substrates and inhibitors of hexokinase (158).

1.7.3 Cyclophilin D

The ability of CsA to potently inhibit induction the MPTP has inevitably drawn the focus to the intracellular receptors of CsA, the cyclophilins.

The cyclophilin family as found in mammalian tissues consists of cyclophilin A (CyPA), a cytosol-derived 18kDa protein, cyclophilin B and C (21kDa) found in the endoplasmic reticulum, and CyPD an 18kDa protein localised to the mitochondria (162-165). The cyclophilins all contain two active sites – a site of endonuclease activity (166) and a site of peptidyl prolyl *cis-trans*-isomerase (PPI-ase) activity (162).

Peptide bonds within proteins can exist in both a *cis* and a *trans* form. During protein folding and refolding, the rate of reaction is frequently determined by the time required for the relevant peptide bond to shift from the *cis* to the *trans* state. Proteins known as peptidyl prolyl *cis-trans*-isomerases catalyse this shift thus speeding up the rate-limiting reaction. Besides the

cyclophilins a further two structurally unrelated families of proteins displaying PPI-ase activity have been identified, the FK506 binding proteins (FKBPs) and the parvulins (162, 167-169). Cyclophilins have a multitude of physiological functions within the cell, being involved in such activities as protein folding (170) and pre-mRNA splicing (171) and function in the regulation of protein complexes such as the oestrogen receptor (172) and the nuclear pore complex (173). The activities of cyclophilins are now also coming into focus in a pathological context. Besides the probable role of CyPD in MPT, cyclophilins are induced as part of the stress response (174), possess endonuclease activity of potential importance in apoptosis (175) and CyPA has proved to be essential for the successful replication of the type 1 HIV virus (176-179).

In summary, the cyclophilin family are involved in a wide variety of intracellular activities. Furthermore, new cyclophilin-like proteins with an array of properties are regularly being discovered (180-182) suggesting that further key functions may yet be attributed to this increasingly important family of proteins.

Perhaps the best-characterised function of the cyclophilins to date is their involvement in cyclosporin A (CsA) mediated immunosuppression. CsA binds specifically to cyclophilins and blocks the activity of calcineurin leading to inhibition of cytokine expression and suppression of the immune response. CsA and its analogues are fungal-derived metabolites that exhibit strong immunosuppressive activity. More recently, derivates such as *N*-methylVal-4-cyclosporin have been produced that, whilst binding to cyclophilins do not suppress the immune response (140). CsA has long since been identified as a potent inhibitor of the MPTP, and the finding that the mitochondrial cyclophilin, CyPD, is the only mitochondrial receptor for CsA (140) has resulted in extensive study of this protein.

The group of Halestrap demonstrated that CsA and several analogues inhibited the mitochondrial cyclophilin enzymatic activity at a potency that correlated with their ability to

block MPTP induction (139), work that has more recently been corroborated by a publication from Nicolli and co-workers (140). These findings have resulted in the proposal that the mitochondrial CyP could be an integral and important part of the MPTP (183) and the subsequent identification of what has since been termed cyclophilin D (184).

As CyPD is a soluble protein contained in the mitochondrial matrix within the bounds of the virtually impermeable inner mitochondrial membrane, its association with the MPTP must logically be via interaction with an inner mitochondrial membrane-bound protein. This concept has been supported by studies showing that factors such as oxidative stress and increased matrix volume promoted CyPD binding to the inner mitochondrial membrane (185, 186). The fact that ANT is located in the inner mitochondrial membrane and had already been marked as a probable component of the MPTP made it an immediate and obvious candidate for CyPD. Experiments with CyPD affinity columns have shown both that ANT alone of all inner mitochondrial membrane proteins binds to CyPD (140, 157) and also that complexes of VDAC-ANT demonstrate extremely potent CyPD binding (159). Additionally, when VDAC-ANT complexes were isolated with hexokinase that binds to VDAC, CyPD was also found in association with the complex. However, in complexes of VDAC-ANT and creatine kinase that binds directly to ANT no CyPD was detected, implying that creatine kinase prevented CyPD binding to ANT (158). Although ANT and VDAC-ANT are both able to form Ca^{2+}-sensitive high conductance channels, the consistent finding that CsA potently inhibits MPTP induction in isolated mitochondria, intact cells and isolated perfused hearts make it extremely probable that CyPD is a genuine and important component of the MPTP.

1.7.4 Other potential regulatory molecules

In addition to those detailed above, a number of other molecules have been identified that may have a regulatory effect on the MPTP. These include the kinases alluded to previously and some members of the Bcl-2 family such as Bax, Bak, Bcl-xL and Bcl-2 (16).

In summary, the studies detailed here demonstrate that function and regulation of the MPTP is a complex, controversial and expanding subject. Although some conflicting results have been obtained and regulatory molecules appear to differentiate widely according to the situation, in is without doubt that a wide variety of molecules must be involved *in vivo*, and more may yet be discovered.

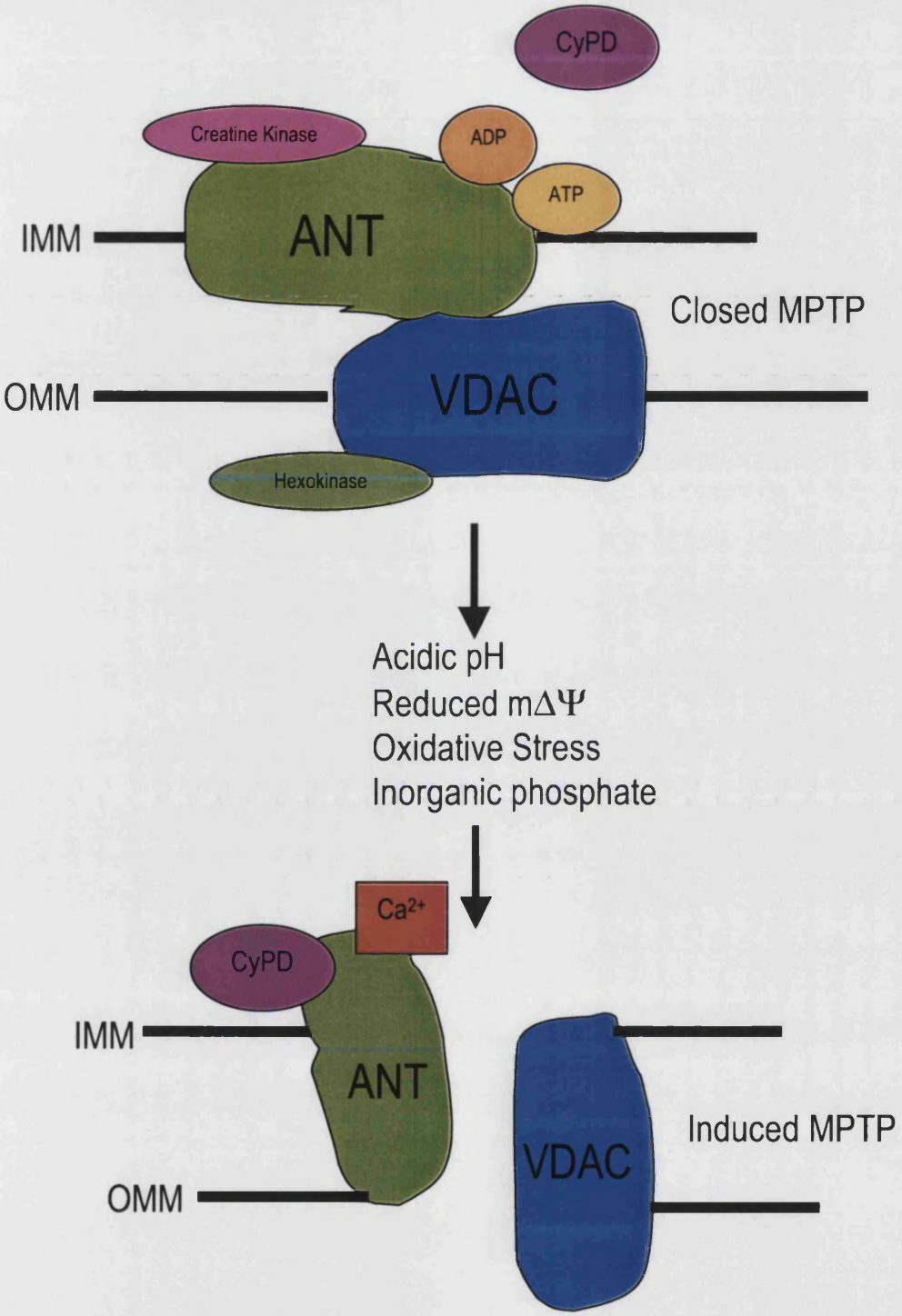


Figure 3: The molecular mechanism and induction of the mitochondrial permeability transition pore (MPTP) is a controversial area. This representation summarises the major studies in the field to give a possible scenario (IMM/OMM: inner/outer mitochondrial membrane; ANT: andenosine nucleotide translocase; VDAC: voltage dependent anion channel; CyPD: Cyclophilin D).

1.8 The molecular mechanism of the mitochondrial permeability transition pore

Induction of the MPTP requires a specific and now well-established set of conditions. Numerous studies have found that the primary trigger for MPTP induction is a high concentration of Ca^{2+} in the mitochondrial matrix. Other factors that are known to affect the sensitivity of the MPTP to Ca^{2+} are oxidative stress, pH, adenosine nucleotide depletion, increased inorganic phosphate and reduced $m\Delta\Psi$ as illustrated in Figure 3.

1.8.1 Matrix Ca^{2+} concentration

The concentration of Ca^{2+} in the mitochondrial matrix has long been considered the most important factor determining induction of the MPTP. Numerous reports as detailed above have shown that a high concentration of matrix Ca^{2+} can induce the MPTP in isolated mitochondria. Some studies have also shown that a high enough Ca^{2+} concentration can cause MPT even in the presence of the classical MPTP inhibitor, CsA (187-189). The close relationship between matrix Ca^{2+} concentration and MPTP induction was effectively highlighted by Al-Nasser and Crompton who published work showing matrix Ca^{2+} dependent transient opening and closing of the MPTP in isolated liver mitochondria. Induction of the MPTP was demonstrated to be proportional to the matrix Ca^{2+} concentration and independent of $m\Delta\Psi$ (127). Subsequently, it has been proposed that the binding of Ca^{2+} to the ANT may initiate a conformational alteration, causing it to form a channel (183). There is supporting evidence that Ca^{2+} binds to a very specific site of action on the matrix ANT surface, as Ca^{2+} induced MPT is effectively inhibited by other divalent cations (190).

1.8.2 Adenosine nucleotides and inorganic phosphates

The potent inhibitory effect of adenosine nucleotides on the MPTP has frequently been demonstrated. As the major function of the ANT is to transport adenosine nucleotides across

the inner mitochondrial membrane, the high affinity binding of adenosine nucleotides to the ANT is a well-established fact.

Studies using both reconstituted MPTP complexes and isolated mitochondria have suggested that ADP is a far more potent inhibitor of MPT than ATP (158, 189). A study of purified reconstituted ANT showed that physiological concentrations of ADP inhibited Ca^{2+} induced MPTP-like activity (154). This demonstrates that the inhibitory effect on ADP on the MPTP is almost certainly mediated through the ANT binding site. The authors in fact make the point that the conversion of ANT to a non-specific channel can only take place at low adenosine nucleotide levels. During ischaemia-reperfusion, such depletion of adenosine nucleotides is in evidence (191-194) improving the likelihood of MPTP induction. In addition, ADP binding to ANT is antagonised by mitochondrial membrane depolarisation and by oxidative stress (189), both of which are common criteria of reperfusion injury.

An obvious consequence of adenosine nucleotide depletion is a corresponding build up of inorganic phosphates. It has been demonstrated that the accumulated intracellular phosphate may have the effect of lowering the matrix ADP content, thus removing ADP inhibition of the MPTP (195, 196). This proposition may account for repeated reports of inorganic phosphate induced activation of the MPTP (143, 144, 197). However it is interesting to note that an additional study by the same group has shown an inhibitory role for low concentrations of inorganic phosphate, due to its reported ability to complex matrix Ca^{2+} (198). This suggests that physiological concentrations of phosphates could have an inhibitory effect on the MPTP that is removed by the onset of ischaemia.

1.8.3 Acidic pH

The onset of ischaemia results in an acidic pH that has been shown to inhibit the pore, although the precise mechanism of this inhibitory effect is not yet apparent (127, 190, 197).

Bernardi and co-workers have published data showing that H^+ may modulate the binding of CyPD to ANT by affecting a specific ANT histidine residue (190, 199). However, Halestrap and colleagues have found no evidence for the effect of acidic pH on CyPD binding and instead speculate that H^+ may compete with Ca^{2+} for the same binding site on the ANT therefore reducing the sensitivity of the MPTP to Ca^{2+} (157, 186).

Whatever the mechanism, it is generally accepted that upon reperfusion the pH increases again moving the inhibition and allowing opening of the pore (190, 200). However a recent report showed that in energised mitochondria, an acidic pH caused MPTP induction due to increased uptake of inorganic phosphate. The authors therefore suggest that acidosis during ischaemia and reperfusion may increase MPTP induction and exacerbate tissue damage (197).

1.8.4 Oxidative Stress

There is increasing evidence to show that agents of oxidative stress are also potent inducers of the MPTP. A study by Takeyama and colleagues showed that damage sustained by mitochondria on exposure to free radicals was completely inhibited by CsA, suggesting that the presence of free radicals triggered induction of the MPTP resulting in mitochondrial damage (201). Other studies in isolated mitochondria have also shown that agents of oxidative stress can trigger Ca^{2+}-dependent inner mitochondrial membrane permeability transition (143, 202) that can be inhibited by CsA and bongkrekic acid (143, 148). Studies of oxidants have shown that they impede ADP inhibition of MPTP induction (189, 203) and a comparison of oxidative agents with thiol reagents has suggested that oxidants may modify cysteine residues on the ANT. This could be expected to antagonise the binding of ADP and possibly also CyPD, thereby sensitising the MPTP to the action of Ca^{2+} binding (189).

1.8.5 Mitochondrial transmembrane potential

Bernardi and colleagues have produced substantial evidence to show that the likelihood of MPTP induction is increased as the mitochondrial transmembrane potential ($m\Delta\Psi$) decreases (125). The group then followed up these findings by demonstrating that oxidation of critical thiol groups contained in the MPTP increases the probability of its induction despite a high $m\Delta\Psi$ (204, 205). Halestrap and colleagues have since extended this work, producing evidence that these thiol groups are situated on the ANT itself, in the vicinity of the matrix adenosine nucleotide binding site. Based on the electrically driven nature of ATP and ADP exchange by the ANT, the group has hypothesised that a high $m\Delta\Psi$ increases the affinity of the matrix adenosine nucleotide binding site for ADP, thus effecting a strong inhibition on the MPTP (189).

1.9 The role of cyclophilin D

As previously detailed, recruitment of CyPD to the inner mitochondrial membrane has been demonstrated under conditions known to provoke opening of the MPTP. In addition to this, numerous studies have revealed potent inhibition of the MPTP by the CyP-binding CsA and a corresponding protection from reperfusion-linked damage. Thus, it is now generally accepted that CyPD is probably an important component of the MPTP that encourages its induction through binding to ANT.

Bernardi's group shows that CyPD is detectable both in the mitochondrial matrix and associated with the inner mitochondrial membrane suggesting that under normal conditions only a fraction of CyPD is associated with ANT (140). Crompton has also presented evidence that at least some CyPD is physiologically associated with the inner mitochondrial membrane (160). Halestrap showed that various agonists that encourage MPT also increased recruitment and binding of CyPD to the inner mitochondrial membrane (185, 186). Taken together, these findings are in agreement with a model of pathological conditions promoting CyPD binding to ANT in order to influence the formation of the MPTP, an entity that may also function physiologically.

However, the issue of exactly how and where CyPD binds to ANT and influences the functioning of the MPTP is a point as yet unsettled. A number of conflicting reports from some of the key workers in the field have given rise to variant theories.

Halestrap has produced evidence that CyPD may bind to ANT on the matrix surface at a specific proline residue - Pro^{61} (189). This proposal is based on the finding that cross-linkage of an adjacent cysteine residue enhances CyPD binding (189) and on the assumption that CyPD binds via its active site supported by the ability of CsA to prevent CyPD binding to ANT

(157). It is additionally sustained by evidence that three yeast-derived ANT isoforms that lack this proline residue are also lacking in detectable CsA-sensitive MPTP-like activity (189). Conversely, the finding of Crompton's group that CsA treatment was unable to prevent CyPD from binding to VDAC-ANT complexes, despite inhibiting the MPTP-like activity of the complex led them to propose that CyPD binds via a different point remote from its active site allowing CsA binding (159). Instead the group proposes a complex of VDAC-ANT-CyPD that flickers between an open and closed state according to matrix concentrations of Ca^{2+}.

This however, is in disagreement with findings from the previously alluded to CyPD affinity column study of Woodfield and colleagues. In addition, this investigation reports the effect of CAT and BA, both of which stabilise ANT in one of its two alternative conformations (157). The effect of both of these agents was to produce a significant decrease in the amount of ANT that bound to CyPD. Thus the authors suggested that the binding of CyPD could potentially induce a conformational change in ANT that increases the binding affinity.
However, this last point is in conflict with an increasing amount of evidence that suitably high levels of matrix Ca^{2+} can induce the MPTP irrespective of the presence of CyPD or the inhibitory action of CsA. As previously mentioned, there is now strong evidence that purified, reconstituted ANT can form a Ca^{2+}-sensitive channel (153, 154). In addition, several reports in isolated heart and liver mitochondria have demonstrated that the inhibitory effect of CsA on the MPTP can be overcome by high matrix Ca^{2+} concentrations (187-189).

1.9.1 The influence of CsA

The last point also raises the equally uncertain issue of how the inhibitory effect of CsA is mediated.

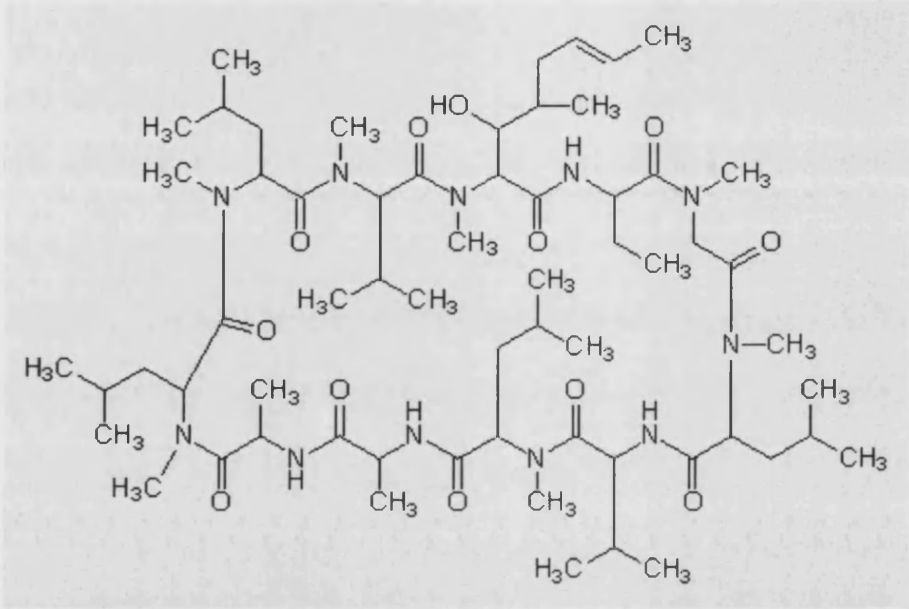


Figure 4: The molecular structure of cyclosporin A ($C_{62}H_{111}N_{11}O_{12}$)

As CyPD is an often-cited component, or at least regulator, of the MPTP, this provides an obvious reason for the ability of CsA to inhibit induction of the pore. As molecules exhibiting PPI-ase activity are able to induce structural alterations in their substrates, a frequent postulation has been that the PPI-ase activity of CyPD may be instrumental in its influence over ANT and the induction of the MPTP. This leads to the reasonable assumption that CsA mediates its effect on the MPTP by inhibiting CyPD PPI-ase activity. Publication of the crystalline structure of the CsA-CyP complex has since confirmed that CsA binds to and blocks the active PPI-ase site of CyPs. This occurs with some structural change in CsA but very little alteration of CyPD (206). Despite this however, studies that have been published to date have failed to completely clarify the mechanism of CsA inhibition.

A report by Bernardi's group showing that CsA caused the release of CyPD from preparations of mitochondrial membranes led them to propose that CsA binding causes conformational changes in CyPD causing its dissociation (140). However, Crompton and colleagues then contradicted this finding in a study that demonstrated the inability of CsA to prevent the binding of VDAC-ANT complexes to a CyPD affinity column (159). Furthermore, both studies are in disagreement with Woodfield and colleagues who found that the pre-incubation of a CyPD affinity column with CsA successfully prevented ANT binding and moreover that VDAC, although present did not bind (157). In the case of the first report, no MPTP-like activity could be detected in the submitochondrial particles under study, suggesting some functional abnormality in the mitochondrial proteins that may have allowed CyPD displacement by CsA. The discrepancies between the last two reports may be due to differences in methodology as the authors have in fact suggested (207, 208). However, as Crompton and colleagues showed the VDAC-ANT-CyPD complexes to be functionally active, it is also reasonable to suggest that in this situation, the binding of VDAC could produce a structural alteration in ANT that allows it to bind CyPD despite the presence of CsA. Furthermore, CsA binding successfully inhibited the MPTP-like activity, as is the case in isolated mitochondria and intact cells.

This raises the possibility that CsA could potentially act in one of two ways. Binding to CyPD may completely prevent its association with ANT making it less sensitive to Ca^{2+} as endorsed by Halestrap. Alternatively, CsA binding to CyPD that is already in association with ANT may act purely as a physical barrier, either obscuring the Ca^{2+} and Pi binding sites and therefore increasing the threshold level of matrix Ca^{2+} required for MPTP induction or blocking the passage of larger substances but allowing the movement of ions as suggested by Crompton (159). This is supported to an extent by a previous study of Broekemeier and colleagues that

showed efficiency of CsA inhibition irrespective of whether application was prior to or after the MPTP inducing agent (209).

Ultimately, with the publication of so many conflicting pieces of evidence, the mechanism of MPTP induction and the action of CsA may not be finally resolved without definitive elucidation of the three-dimensional structure of at least part of the complex. In addition, many and varied claims have been made for the action of CsA within the cell meaning that complications may well arise when knowledge gained *in vitro* in tested *in vivo*.

The best-characterised action of CsA to date besides inhibition of the MPTP through CyPD binding is its immunosuppressive effects via calcineurin inhibition. CsA is believed to bind to cyclophilin A (CyPA) with these complexes then associating with calcineurin to inhibit its phosphatase activity. This in turn prevents the dephosphorylation and consequential nuclear import of NFAT (nuclear factor of activated T-cell) transcription factors thus blocking the pathway leading to the expression of immune response cytokines in the T-cell. This same signalling pathway is present in cardiac cells and, when activated, leads to the transcription of hypertrophic genes. This has therefore suggested a preventative role for CsA in hypertrophic heart disease. However, although a substantial number of studies have been carried out in both cultured cardiomyocytes and rodent models of cardiac hypertrophy, the experimental data in support of this hypothesis is conflicting. Some studies have demonstrated almost complete attenuation of cardiac hypertrophy by CsA whereas other data has been completely negative. In fact there is some evidence to suggest that CsA could even exacerbate hypertrophy and it certainly has other unwanted side effects such as secondary hypertension (reviewed in 210, 211). A number of possible explanations for the conflicting data have been put forward including model-specific effects, genetic sensitivity to CsA and side effects resulting from systemic administration of CsA (210), but no consensus has yet been reached.

Other calcineurin-independent effects have also been attributed to CsA including inhibition of some mitogen-activated protein kinases (MAPKs), long-term effects on the sarcoplasmatic reticulum Ca^{2+}-release channel and on L-type Ca^{2+} channels and immunosuppressive activity through TGFβ1 (reviewed in 210). CsA has also been shown to induce apoptosis in various tissues (212-215). It is widely accepted that CsA binds specifically to members of the cyclophilin family and, as yet, no other intracellular targets have been proposed. All of the effects mentioned above are therefore assumed to be a consequence of CsA binding to cyclophilins within the cell.

1.10 Aims and hypothesis

Although the molecular structure of the mitochondrial permeability transition pore (MPTP) and the mechanism of its induction are by no means fully elucidated, the majority of the evidence highlights an important functional role for cyclophilin D. Therefore the studies reported in this investigation attempted to draw together several strands of research with the aim of: (i) examination of the induction of the MPTP in response of a variety of pathological stimuli in neonatal primary cardiomyocytes, which may offer a more authentic and valuable insight than an established cell line; (ii) investigation of the effect of MPTP induction on an array of important parameters of cell viability; (iii) exploration of the role of cyclophilin D in MPTP induction; (iv) study of relevant parameters in an *in vivo* model of ischaemia-reperfusion injury.

The hypothesis upon which this investigation is founded is:

Induction of the mitochondrial permeability transition pore plays an important role in cardiac ischaemia-reperfusion injury. Inhibition of molecular components of the pore such as cyclophilin D therefore offers protection to cardiac tissue against ischaemia-reperfusion injury.

2: Materials and Methods

2.1 Materials

All chemicals were obtained from Sigma (UK) unless otherwise stated. Cell culture reagents and plates were purchased from Life Technologies (Gibco). HPLC-grade water was purchased from Fisher. Cyclosporin A, propidium iodide, bongkrekic acid and FK506 were obtained from Calbiochem.

2.2 Culture and treatment of neonatal primary cardiomyocytes

2.2.1 Preparation of primary cardiomyocytes

Primary rat neonatal cardiomyocytes were isolated by collagenase/ pancreatin digestion using 1-3 day old (Sprague-Dawley) neonate hearts according to the method of Chien and colleagues (216).

Neonates were killed by rapid decapitation, rinsed in 70% ethanol and placed in petri dishes. A midline incision was made through the sternum and the heart was excised using forceps. The hearts were then trisected and placed in sterile ADS buffer (116.4mM NaCl, 15.8mM HEPES, 0.9mM NaH_2PO_4, 5.5mM Glucose, 5.4mM KCl and 0.8mM $MgSO_4$ at pH 7.35) on ice and transferred to a sterile cell culture environment.

The hearts were then digested for 7 min at 37°C in an enzyme solution consisting of 30mg Type II collagenase (Worthington Biochemical Corporation) and 0.6mg/ml pancreatin (GibcoBRL) in 7ml of ADS buffer. As the heavier cardiac material settled in the bottom of the tube the supernatent, at this point containing mostly blood cells and debris, was easily removed using a pipette, discarded, and the digestion was repeated once.

To obtain cardiomyocytes, the hearts were then subjected to further digestion in 7ml of the same enzyme solution for 15 min at 37°C. The solution removed from the hearts after this digestion was retained, added to 2ml foetal calf serum (FCS) and centrifuged at 1000rpm for 6 min. The supernatant was discarded and the cells were retained in 4ml FCS at 37°C. The hearts were digested for 15 min a further 5 times and the cardiac cells obtained were pooled and centrifuged at 1000rpm for 6 min.

The pellet of cardiac cells was then resuspended in plating media consisting of 68% Dulbecco's Modified Eagles Medium (DMEM), 17% Medium 199 (M199), 100U/ml penicillin/streptomycin (Life Technologies), 10% horse serum and 5% FCS at a concentration of approximately 10ml media per 6 neonate heats used. This suspension was pipetted into

standard 10ml tissue culture dishes and incubated at 37°C for 45 min. During this period contaminating fibroblasts attach to the petri dished while myocardial cells remain suspended in the media. These unattached cells were then removed and plated at a concentration of $1x10^6$ /ml plating media into culture dishes pre-coated with 1% gelatin in Dulbecco's phosphate buffered saline (PBS). After 24 hours, the media was changed for a low-serum maintenance media consisting of 80% DMEM, 10% M199 and 1% FCS.

Experiments were conducted approximately 48hr after initial plating using spontaneously beating cardiomyocytes.

2.2.2 Induction of apoptosis in primary cardiomyocytes

Primary cardiomyocytes were treated with the widely recognised apoptosis inducer staurosporine (SSP) for up to 24hr. SSP was reconstituted in 50% methanol, diluted to its' final concentration (5μM) in maintenance media and applied to the cardiomyocytes.

2.2.3 Induction of necrosis in primary cardiomyocytes

Necrotic cell death was induced by addition of t-butylhydroperoxide (t-BOOH) for 1hr. t-BOOH was purchased as a 70% stock solution, diluted to its' final concentration (100μM) in maintenance media and applied to the cardiomyocytes.

2.2.4 *In vitro* simulation of ischaemia-reperfusion injury

To subject the cardiomyocytes to conditions of simulated ischaemia and reperfusion, the culture media was removed and replaced with a solution based on a protocol of Esumi and colleagues (217) incorporating modifications by Dr.R.Heads (personal communication). This consisted of 137mM NaCl, 15.58mM KCl, 1.2mM $MgCl_2.6H_2O$, 2.5mM $CaCl_2.2H_2O$, 20mM 2-deoxyglucose, 20mM Na lactate and 1mM sodium dithionite This solution in combination with a

hypoxic environment was designed to simulate the conditions of *in vivo* ischaemia by replicating the approximate concentrations of potassium, hydrogen and lactate ions and by the presence of 2-deoxyglucose to inhibit glycolysis and sodium dithionite to scavenge oxygen and inhibit mitochondrial respiration. The cells in this solution were then placed in a hypoxic chamber at 37°C for up to 3 hours. The chamber was perfused with 95%N_2 and 5% CO_2 at a flow rate of 5 litres/hr for 1 hr and thereafter at a rate of 1.5litres/hr resulting in a pO_2 of 2mmHg.

Upon removal from the chamber the cardiomyocytes were either analysed immediately, or reperfused in culture media for up to 24 hours prior to analysis.

2.3 Analysis of cell viability

2.3.1 Electron microscopy

All electron microscopy was performed by Mrs. Padmini Sarathchandra.

Primary cardiomyocytes cultured on petri dishes as described above were washed briefly with 0.1M phosphate buffer and fixed in 3% (v/v) glutaraldehyde (Agar Scientific Ltd, UK) in 0.1 M phosphate buffer for 2 hours. After two buffer washes, the secondary fixation was performed with 1% (w/v) osmium tetroxide (Agar Scientific Ltd, UK) in 0.1 M phosphate for one hour at room temperature. Following the secondary fixation, cardiomyocytes were washed twice with distilled water and block stained with 4% (w/v) aqueous uranyl acetate (Agar Scientific Ltd, UK) for one hour in the dark, washed in distilled water and scraped into Eppendorf tubes. The specimens were dehydrated through increasing acetone series and infiltrated with 1:1 acetone: araldite CY212 resin (Agar Scientific Ltd, UK) overnight. After two changes of fresh resin, for a minimum of 3hr each, samples were embedded in araldite CY212 resin and blocks were polymerised at 60°C for 18hr.

Ultra-thin (100nm) sections were cut using a Diatome diamond knife, on a Reichert-Jung Ultracut E ultramicrotome, floated onto distilled water, collected on formvar-coated copper grids and stained with 2% uranyl acetate for 30 min and in lead citrate for 5 min. The stained sections were then viewed on a Jeol 1200EX electron microscope.

2.3.2 Detection of cellular DNA content in primary cardiomyocytes

Cells undergoing apoptosis typically exhibit DNA fragmentation. If these fragments are allowed to diffuse from permeablised cells, then these cells will display a reduced DNA content compared to a control sample (218). Therefore, cardiomyocytes subjected to an inducer of apoptosis were permeablised and analysed for loss of DNA.

Primary cardiomyocytes were harvested using a 0.25% trypsin/EDTA solution and centrifuged at 1500rpm for 5 min. The cells were then fixed in 4% paraformaldehyde/PBS for 30 min, followed by permeabilisation and simultaneous staining of DNA in the hypotonic fluorochrome solution (0.1% sodium citrate, 0.1% X-Triton and 40μg/ml propidium iodide) described by Fried and colleagues (219) for 15 min at room temperature in the dark. The cells were then resuspended in PBS and incubated for a further 15 min in the dark at 37°C to allow the diffusion of small DNA fragments (218) prior to measurement of propidium iodide fluorescence on a flow cytometer (Becton Dickinson FACSCalibar, excitation 488nm, emission 495nm) and analysis of data using CellQuest software.

2.3.3 Detection of LDH release

Quantification of enzyme release from cells with compromised plasma membranes was utilised as a marker of necrosis. A kit for the detection of lactate dehydrogenase (LDH) was obtained from Sigma Diagnostics and media collected from the cardiomyocytes was analysed according to the manufacturer's instructions. The diagnostic kit is based on the ability of LDH to catalyse the reduction of pyruvate according to the following equation,

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{L-Lactate} + \text{NAD}^+$$

The production of NAD^+ results in a decrease in absorbance at 340nm, which is directly proportional to LDH activity. The assay reagent is made by combining 0.4ml of 16.2mmol/L pyruvate with 10ml of 0.194mmol/L NADH in 54mmol/L phosphate buffer at pH 7.5. Addition of 8μl of the sample to 200μl of the reagent starts the reaction. After incubating for 30 seconds, the sample was read at 340nm on a microplate reader (Molecular Devices VERSAmax) and was then read every 60 seconds for 3 min. The average change in absorbance (ΔA) per

minute was calculated and LDH activity was determined for each sample according to the following equation:

$$\text{LDH activity (U/L)} = \frac{\Delta A \text{ per min} \times \text{Total vol.} \times 1000}{6.22 \times \text{Light Path} \times \text{Sample vol.}}$$

2.3.4 Analysis of metabolic activity

Metabolic activity of the cardiomyocytes was assessed by the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2-diphenyl tretrazolium bromide (MTT) reagent by metabolically active cells. The MTT reagent is taken up into the cells where it is reduced, producing purple crystals of MTT formazan (220).

MTT reagent was applied at a final concentration of 500µg/ml PBS. After 30min an equal volume of a solution of isopropanol containing 0.1N HCl and 1% X-Triton was added to stop the reaction and dissolve the crystals. The resulting colour intensity was analysed at 570nm on a microplate reader (Molecular Devices VERSAmax).

2.4 Analysis of mitochondrial transmembrane potential in primary cardiomyocytes

2.4.1 Quantification of mitochondrial transmembrane potential

The decrease in mitochondrial membrane potential was evaluated according to the method established in cells by Zamzami (221). Modifications proposed by Rottenberg were incorporated whereby 3,3'dehexylocarbocyanine iodide ($DiOC_6(3)$) was used at a concentration of <1nM to eliminate membrane quenching resulting in greatly enhanced sensitivity (222). The fluorochrome ($DiOC_6(3)$), due to its cationic lipophilic structure, penetrates freely through membranes into the mitochondrial matrix driven by the $m\Delta\psi$ as a function of the Nernst equation (120). The cardiomyocytes were treated according to the experimental protocol, harvested using trypsin/EDTA, centrifuged at 1500rpm and incubated for 20 min at 37°C in media containing 0.8nM of ($DiOC_6(3)$). The fluorescence levels were measured on a Becton Dickinson FACSCalibar flow cytometer (excitation 488nm, emission 495nm) and induction of the MPTP was quantified by the decrease in fluorochrome binding.

2.4.2 Visualisation of cardiomyocytes by confocal microscopy

Primary cardiomyocytes were cultured on 1% gelatin-coated round, glass coverslips. The cells were washed with and immersed in PBS prior to visualisation on a Carl Zeiss LSM510 inverted confocal microscope system using the manufacturer's own software. 20nM or 0.8nM of ($DiOC_6(3)$) and 1μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added to the cells during visualisation.

2.5 Detection and analysis of Cyclophilin D

2.5.1 Isolation of mitochondria from primary cardiomyocytes

Mitochondria were isolated from primary cardiomyocytes using digitonin according to the method of Trounce and colleagues (223). Primary cardiomyocytes were harvested, centrifuged and washed in separation buffer of pH 7.2 containing 210mM mannitol, 70mM sucrose, 10mM MOPS and 1mM EGTA. The samples were centrifuged at 3000 x g for 5 min, treated with 10% digitonin to permeablise the cells and then homogenized 10 times retaining the cells on ice at all times. After a further centrifugation at 3000 x g, the resulting supernatant was centrifuged twice at 12000 x g for 10min to pellet the mitochondria. All centrifugation was performed at 4°C in a refrigerated centrifuged. The resulting mitochondria exhibited normal ultrastructure as shown in Figure 5.

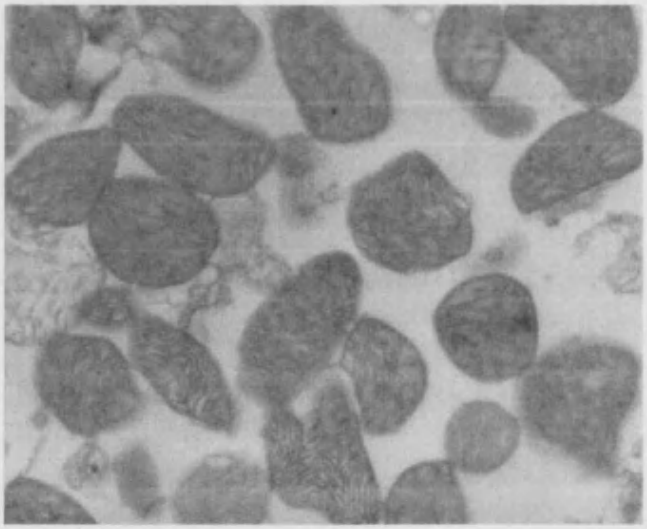


Figure 5: Mitochondria isolated from neonatal primary cardiomyocytes displayed normal ultrastructure with parallel, densely packed cristae and intact membranes (Magnification x 30,000).

2.5.2 Measurement of PPI-ase activity

PPI-ase activity of the mitochondrial fraction was measured by the spectrophotometric method described by Fischer and colleagues (224) incorporating modifications proposed by Kofron and co-workers (225). This method employs the ability of α-chymotrypsin to hydrolyse peptide containing the sequence X-Pro-Phe-pNA for which it has an extremely high affinity. This produces *p*-nitroaniline, which is detectable through its absorbance at 390nm. The rate-limiting step in this reaction is that hydrolysis only occurs when the X-Pro bond is in the *trans* form. Molecules displaying PPI-ase activity such as the cyclophilins have the ability to shift the X-Pro bond from the *cis* to the *trans* form, producing a detectable increase in the rate of the reaction. Prior to measuring PPI-ase activity, the isolated mitochondria were resuspended in isolation buffer containing protease inhibitors and quantified using the Lowry protein concentration assay (Bio-Rad) according to the manufacturer's instructions. An equal concentration of mitochondria was always added to each assay.

35mM HEPES buffer pH 7.8, 0.8mg/ml α-chymotrypsin and isolated mitochondria were combined in a plastic cuvette to a final volume of 990µl and cooled to 4°C. The assay was then initiated by the addition of 10µl of 8mM of the peptide N-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Suc-AAPF-pNA) dissolved in 470mM LiCl/trifluoroethanol (TFE) Recording of the absorbance at 390nm commenced immediately using a Unicam Helios spectrophotometer. Dissolving Suc-AAPF-pNA in LiCl/TFE significantly increases the proportion of the *cis* X-Pro isomers in the solution, thereby ensuring that the *trans* isomers are not all hydrolysed in a "burst phase" during mixing (225).

Absorbance over a 2-minute period was recorded and the rate of reaction (k) was quantified by comparison to a previously constructed standard curve using a data-fitting computer software package (GraFit, Erithacus Software).

The percentage increase in rate, caused by the addition of CyP or mitochondrial fraction, over that of the basic reaction was quantififfed according to the following equation:

$$\% \text{ increase} = \frac{kobs - k0}{k0} \times 100$$

kobs is the rate of reaction with PPI-ase (CyP or mitochondria).

k0 is the rate of reaction with no PPI-ase added.

2.5.3 Synthesis of a specific anti-Cyclophilin D antibody

An antibody specific to Cyclophilin D (CyPD) was synthesised according to the method of Bergsma and colleagues (162). This was however slightly modified to produce a rat-specific antibody (as opposed to human) and therefore the following peptide sequence corresponding to the carboxyl terminus of rat CyPD was synthesised:

D V V K K I E S F G S K S G K

This peptide was synthesised at the Babraham Institute, Cambridge, coupled to the carrier protein keyhole limpet haemocyanin and then utilised to raise specific antibodies in rabbits according to a standard protocol also at the Babraham Institute.

In order to purify the CyPD-specific antibody from the rabbit serum, an immunoaffinity column was prepared using Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. 2ml of Affi-Gel 10 was washed twice with 10ml of cold isopropanol, the excess was removed and 50mg of CyPD-specific peptide dissolved in 1ml dimethyl sulfoxide (DMSO) was added to the gel. The mixture was agitated for 6hr at room temperature after which the gel was washed

again and then any unreacted groups were blocked by washing with an excess of ethanolamine.

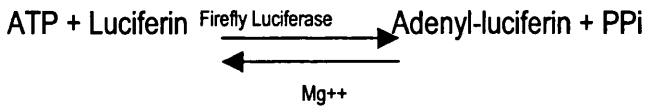
The gel with bound peptide was placed in a glass Econo-Column (Bio-Rad), washed several times by elution with 0.1M sodium phosphate pH 8.0 and then washed one final time whilst 1ml fractions were collected. After 10 fractions were collected, the buffer was changed to 100mM Glycine/HCl buffer at pH 2.2. The change of buffer causes slight structural alterations in the antibodies so they are unable to remain bound and are eluted from the column with the Glycine buffer. After collection of a further 10 fractions, the absorbance of all fractions at 280nm was analysed to determine the location of the antibodies. These fractions were then pooled, returned to a neutral pH by addition of a small amount of 0.1M sodium phosphate pH 8.0 and dialysed in PBS at 4°C overnight.

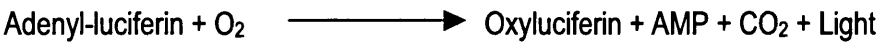
Concentration of the purified antibody was calculated by measurement of protein concentration and comparison to a standard curve of IgG absorbance at 620nm.

2.6 Analysis of ATP production in primary cardiomyocytes

Primary cardiomyocytes grown in 12-well plates and treated as previously described were harvested by trypsin/EDTA, washed twice in PBS and resuspended in 500µl PBS. To ensure consistency of data, the protein content of each sample was determined as previously described and PBS was added so that all samples attained the same protein concentration.

A kit purchased from Sigma Diagnostics was used to determine the amount of adenosine 5'-triphosphate (ATP) released from the primary cardiomyocytes. The system is based on the ability of ATP to interact with luciferin and firefly luciferase to produce light, according to the following equations:





When the amount of ATP is the limiting factor, the light emitted is proportional to the ATP present in the sample.

100µl of ATP Assay Mix (luciferase, luciferin, $MgSO_4$, DTT, EDTA, BSA and tricine buffer salts) was added to each well of a non-transparent microplate and allowed to stand for 3 min. Meanwhile, 100µl ATP releasing agent was added to each well of another microplate along with 50µl dH_2O and 50µl of primary cardiomyocytes suspended in PBS. 100µl of this mixture was then added to the ATP Assay Mix using a multi-channel pipette and after precisely 10 seconds, the amount of light emitted was measured.

In order to calculate the ATP released in each instance, an internal standard was required for each sample. This was achieved measuring a second 50µl aliquot of each sample, replacing the 50µl dH_2O with 50µl ATP of a known concentration. The ATP content of each sample could then be calculated according to the following equation where SAM is sample, IS is internal standard and ATP is measured in moles:

$$ATP_{(SAM)} = \frac{ATP_{(IS)} \times Light_{(SAM)}}{Light_{(SAM+IS)} - Light_{(SAM)}}$$

2.7 Measurement of free radical activity

Free radical activity in primary cardiomyocytes was detected using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) obtained from Molecular Probes. This is a widely used probe which upon diffusion into the cell, is hydrolysed by intracellular esterases to produce 2',7'- dichlorodihydrofluorescein which is then oxidised by intracellular free radicals resulting in fluorescence which is detectable on a flow cytometer.

Primary cardiomyocytes were cultured and treated as described previously. H_2DCFDA was dissolved in DMSO, diluted in Maintenance Media to a final concentration of 500nM and added to the cells simultaneously with the inducer of cell death (e.g. SSP). The cardiomyocytes were incubated in the dark prior to harvesting using 0.25% trypsin/EDTA, 2 washes in PBS and analysis on a flow cytometer (Becton Dickinson FACSCalibar: excitation 488nm, emission 495nm) during which the samples were shielded from direct light.

2.8 Immunochemical Assays

2.8.1 Western blotting

Primary cardiomyocytes were scraped directly into Laemmli Sample Buffer containing 5% β-mecaptoethanol. Mitochondrial pellets isolated from adult rat cardiac tissue were resuspended in the same and all samples were boiled for 10 min to denature the proteins.

25µl of each sample was run at 200V on a 12% Tris-HCL Ready Gel (Bio-Rad) and transferred to Hybond ECL Nitrocellulose membrane (Amersham Life Science) using the Bio-Rad Mini-PROTEAN II system according to the manufacturers instructions.

The membrane was blocked for 1hr in PBS containing 5% Marvel and 0.02% Tween 20 and washed briefly in PBS. It was then sealed in a plastic bag containing 2ml anti-CyPD antibody and shaken vigorously for 1hr. Following 3 x 10 min washes with PBS containing 0.02% Tween (PBST), the membrane was then immersed in 5ml of the secondary antibody (swine anti-rabbit HRP-linked) at a 1:1000 dilution and shaken for 1hr. After a further 3 x 10 min washes in PBST, the membrane was immersed for 2 min in equal amounts of ECL detection reagents 1 and 2 (Amersham Life Science), enclosed in Saran wrap and exposed to X-ray film in the dark.

2.8.2 ELISA assay

CyPD peptide (see 2.5.3) was dissolved in PBS to a concentration of 0.5mg/ml and 10µl was added to the wells of a 96-well Immuno-plate (Life Technologies). The plate was then incubated at 4°C overnight. The wells were washed 3 times with PBST and then blocked with 200µl of 2% Marvel/PBS at 4°C for 2 hr. Dilutions of each sample were made at 1 in 50, 200, 800, 3200 and 12800 and 50µl of each dilution were incubated in triplicate for 1 hr at room temperature. The plate was then washed 3 times in PBST and 50µl of a 1 in 500 dilution of swine anti-rabbit horseradish peroxidase antibody (Dako) was applied to all wells for 1 hr.

The plate was then washed again as previously and 100µl of 0.1M sodium phosphate pH 6.0 containing 2mg/ml o-Phenylenediamine Dihydrochloride with 1µl/ml H_2O_2 was added to all wells. After approximately 6 min to allow colour development, the absorbance was read at 450nm using a microplate reader (Molecular Devices VERSAmax).

2.9 Measurement of protein concentration

Protein concentration was measured using the Bio-Rad *DC* Protein Assay, which is itself based upon the original Lowry method. When protein in the sample is reacted with an alkaline copper tartrate solution, the protein is then able to reduce Folin reagent resulting in development of a blue colour with maximum absorbance at 750nm. The assay was conducted in 96-well microplates according to the manufacturer's instructions as follows.

A working reagent was prepared by adding 20µl of Reagent S (a detergent) to every 1ml of alkaline copper tartrate solution used. 25µl of this working was added to 5µl of each of the samples in a microplate and 200µl of Folin reagent was added to all. After mixing, the colour was allowed to develop for 15 min prior to measuring absorbance at 750nm using a microplate reader (Molecular Devices VERSAmax).

2.10 Model of cardiac ischaemia-reperfusion injury in adult Sprague-Dawley rats

2.10.1 Surgical procedure

All animal surgery was performed by Mr. Colin Shurey in strict accordance with Home Office regulations under licence number 70/4780.

An adult female Sprague-Dawley rat of 10-12 weeks old was anaesthetised with Hypnorm and Diazepam and ventilated at 4ml/breath, 30 breaths/minute.

A short incision was made downwards from the xiphisternum, the abdominal muscle was incised and the rat was dissected bilaterally to the base of the ribcage. From these lateral points the skin was then incised up to the axillae and the diaphragm was divided. The ribs were then divided so that the ribcage could be retracted back over the head, thus exposing the heart.

A 7/0 silk suture was passed around a major branch of the left coronary artery – the Left Anterior Descending (LAD) with at least a 1mm gap either side of the vessel and tightened in order to occlude the vessel. To reperfuse, the suture was loosened to allow the normal flow of blood to resume.

To confirm successful LAD occlusion, the suture was tightened again and 2ml Evans Blue dye was injected into the bloodstream via a femoral vein. All areas with normal flow of blood were infiltrated by the blue dye whilst a significant section of the left ventricle was excluded. This area of tissue was excised and taken for analysis.

Control rats were subjected to the same procedure excepting tightening of the suture and injection of Evans Blue dye. A section of the left ventricular wall was then excised for analysis.

2.10.2 Preparation of cardiac tissue samples for electron microscopy

Samples of cardiac tissue were excised from the left ventricular wall and fixed in 0.1M phosphate buffer containing 3% (v/v) glutaraldehyde (Agar Scientific Ltd, UK) in 0.1 M

phosphate buffer for 2 hours. After two buffer washes, a secondary fixation was performed with 1% (w/v) osmium tetroxide (Agar Scientific Ltd, UK) in 0.1 M phosphate for one hour at room temperature. The samples were then dehydrated through an increasing acetone series and infiltrated with 1:1 acetone: araldite CY212 resin (Agar Scientific Ltd, UK) overnight. After two changes of fresh resin, for a minimum of 3hr each, samples were embedded in araldite CY212 resin and the resulting blocks were polymerised at 60°C for 18hr.

Ultra-thin (100nm) sections were cut using a Diatome diamond knife, on a Reichert-Jung Ultracut E ultramicrotome, floated onto distilled water, collected on formvar-coated copper grids and stained with 2% uranyl acetate for 10 min followed by Reynolds lead citrate for 10 min. The stained sections were then viewed on a Jeol 1200EX electron microscope.

2.10.3 Assessment of plasma LDH activity

0.5ml blood samples were taken from Sprague-Dawley rats via a femoral vein. The samples were then centrifuged at 1500rpm to remove blood cells and the plasma was stored at –70°C prior to measurement of LDH activity by the method described in section 2.3.3.

2.10.4 Isolation of mitochondria from the left ventricle

The left ventricle was excised from the adult rat heart and placed in chilled isolation buffer containing 1mM EGTA. The isolation buffer consisted of 225mM Mannitol, 75mM Sucrose, 10mM MOPS, 10mM Tris Base in HPLC grade water, pH 7.2 - 7.25 at 4°C. The cardiac tissue was then minced with scissors and briefly homogenised whilst being maintained on ice at all times. The homogenate was then centrifuged at 600g for 5 min followed by a further centrifugation of the supernatant at 1000g for 5 min. The resulting supernatant was then centrifuged at 8800g for 10 min and the mitochondrial pellet was resuspended in isolation

buffer with no EGTA. The mitochondria were then washed twice at 8800g for 10 min and resuspended in a small amount of buffer suitable for the intended analysis.

The resulting purified mitochondria exhibited normal ultrastructure as shown below in Figure 6.

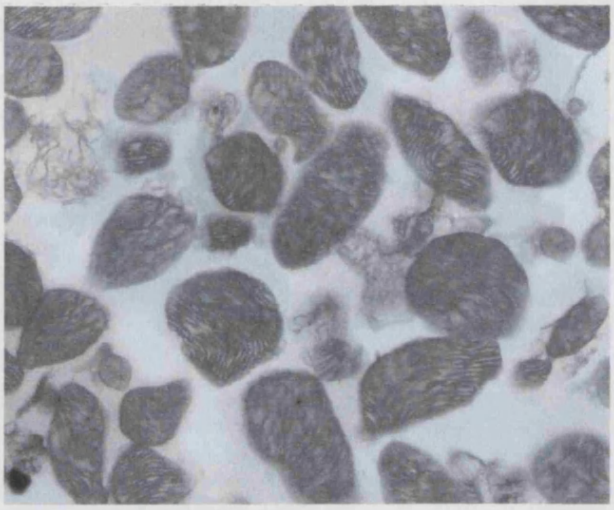


Figure 6: Mitochondria isolated from adult Sprague-Dawley cardiac tissue displayed normal ultrastructure with parallel, densely packed cristae and intact membranes (Total magnification x 30,000).

2.10.5 Assessment of $m\Delta\Psi$ by the retention of tetraphenylphosphonium

An electrode system for the accurate detection and quantification of tetraphenylphosphonium (TPP^+) was kindly provided and set up by Professor A. Panov of the Department of Neurology, Emory University School of Medicine. The TPP^+-sensitive electrode made by Prof. Panov, is itself filled with 10mM TPP^+. The reference electrodes were commercially available Ag/AgCl electrodes and connecting tubes were filled with 3M KCl. The electrodes were connected to a transformer and the data was amplified and recorded and stored on computer. All hardware

and software was purchased from C&L Co, Pennsylvania except the amplifier/filter 202A from Warner Instrument Corp. USA.

TPP^+ is taken up into mitochondria proportionally to the $m\Delta\Psi$ - the higher $m\Delta\Psi$, the more TPP is absorbed. In this system of electrodes, the presence of TPP^+ in the solution surrounding the electrode creates an increase in voltage that can be converted to give the mean $m\Delta\Psi$. When Ca^{2+} is added to the medium it is taken up into the mitochondrial causing an increase in matrix Ca^{2+} concentration and a consequential reduction in $m\Delta\Psi$. The higher the initial value of $m\Delta\Psi$ is the more Ca^{2+} can be added prior to depolarisation.

The tips of the TPP^+ and reference electrodes were lowered into 2ml of Vercesi medium (200mM sucrose, 25mM KCl, 3mM Gly-Gly, 2mM K_2HPO_4 and 1mM $MgCl_2*6H_2O$) containing a magnetic stirrer, which was gently activated for the duration of the experiment. 10mM succinate was added and recording of data immediately commenced. 10µl of 0.1mM TPP^+ was added 3 times at 30 second intervals in order to provide calibration for the experiment and a suspension of mitochondria from the left ventricles of 2 Sprague-Dawley rats was added to a final concentration of 1mg/ml. This caused a sharp decrease in measurable TPP^+ due to its uptake by the mitochondria. After 1 min 5 µl of 10mM Ca^{2+} was added at 1-minute intervals, this resulted in a steady increase in measurable TPP^+ and Ca^{2+} addition continued until TPP^+ levels became constant showing that full depolarisation had occurred.

$m\Delta\Psi$ was calculated according to the following equation:

$m\Delta\Psi$ (-mV) = (RT/nF) x [TPP^+] inside mitochondria / [TPP^+] outside mitochondria

Where:

R = gas constant T = room temperature

n = charge number F = Faraday constant

The actual equations used were those presented by Kamo and colleagues (226) with additional calculations to allow for TPP^+ binding to the mitochondrial membranes according to Jensen and co-workers (227). The binding constants for cardiac derived mitochondria were taken from a publication of LaNoue and colleagues (228).

2.11 Statistical Analysis

All data were analysed using a statistical package associated with Graphpad Prism software. One-way analysis variance was used with the Bonferroni post-test to compare multiple groups. All primary cardiomyocyte experiments were performed in replicates of 4 and repeated using cardiomyocytes from 3 separate cultures.

3: Apoptosis, Necrosis and Simulated Ischaemia: The Effect of MPTP Inhibition

3.1 Introduction

As ischaemia-reperfusion injury is such a common cause of injury in many solid tissues, the type of cell death occurring in this situation is clearly an important factor in implementing any preventative measures. Until comparatively recently all cell death due to ischaemia in solid tissues was believed to take the form of coagulative ischaemia, a common form of ischaemia in which the area of tissue affected retains its basic structure for some time following the death of the cells. Recently however, the role of apoptosis in cardiac tissue has generated much interest due to experimental evidence of it's possible involvement in both ischaemia-reperfusion injury and muscle fibre slippage following an infarction (45, 55, 229-232).
As previously mentioned, there are many conflicting reports concerning the nature of cell death in cardiac injury and the involvement of apoptosis is a contentious issue. This is further exacerbated by the emerging opinion that apoptosis in pathological situations may be an extreme example of cell death as opposed to a distinct process. As well as complicating the already contentious issue of quantification of cell death, this also questions the wisdom of applying specific inhibitors of apoptosis to pathological situations.

Induction of the mitochondrial permeability transition pore (MPTP) is demonstrably involved in both apoptosis and necrosis and has been envisaged as a therapeutic target irrespective of the mechanism of cellular injury. In this chapter therefore, the effect of the MPTP inhibitor Cyclosporin A (CsA) was investigated in 3 models of cardiomyocyte death.

3.2 Objective

The aim of this project was to establish models of apoptotic, necrotic and ischaemic cell death in neonatal rat primary cardiomyocytes and to evaluate the effect of MPTP inhibition in each situation.

The purpose of the study was to determine whether inhibition of the MPTP could rescue cardiomyocytes from death regardless of the pathological process.

3.3 Materials and Methods

Neonatal primary cardiomyocytes were isolated and cultured as described in chapter 2.2.1. Cell death was induced using staurosporine (SSP), t-butylhydroperoxide (t-BOOH) and a solution designed to replicate the extracellular conditions of ischaemia-reperfusion injury as detailed in chapter 2.2.2-4.

A range of methods as described in chapter 2.3 was then employed to quantify the resulting cell death.

3.4 Results

3.4.1 Neonatal primary cardiomyocyte cultures

Neonatal rat primary cardiomyocytes were grown in 24 or 12-well plates as appropriate to the experimental procedure. The cultured cells formed a spontaneously beating monolayer, while some dead cells that did not adhere, remained attached to the live cells despite repeated washes (Figure 7). The adhered cells appeared healthy when analysed by electron microscopy (EM) as shown in Figure 8a & b. Occasional fibroblasts could be seen in the cultures as pictured in Figure 8c. As fibroblasts in primary culture are able to grow and multiply, they became more numerous as the culture aged. Therefore experiments were always performed 48hr after plating.

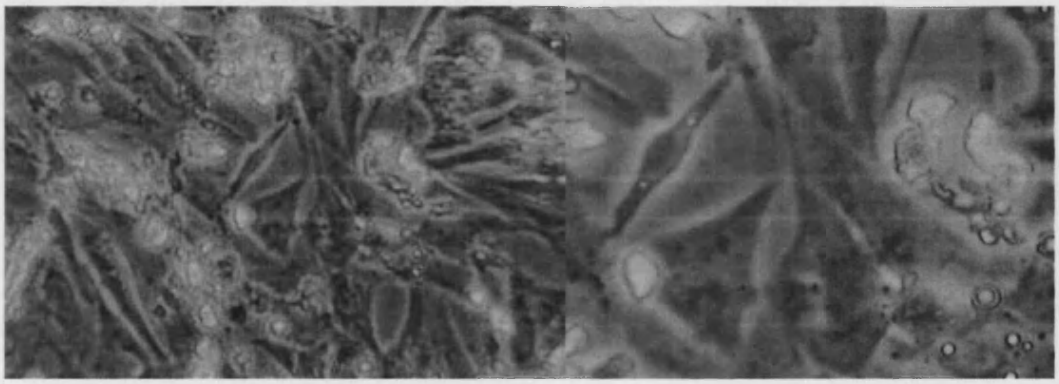


Figure 7: Cultured neonatal primary cardiomyocytes 48hr after initial plating.

These photographs of primary cardiomyocytes visualised by phase-contrast show a monolayer of dark adhered cells that were beating at the time of photography. The small bright cells are those that have died after failing to adhere but remain attached to the cultures (Total magnification x 400 [left image] and x 1200 [right image]).

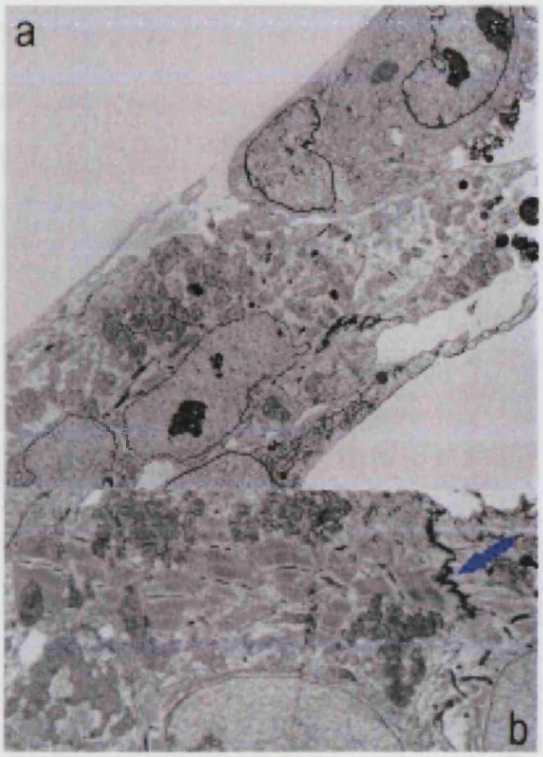
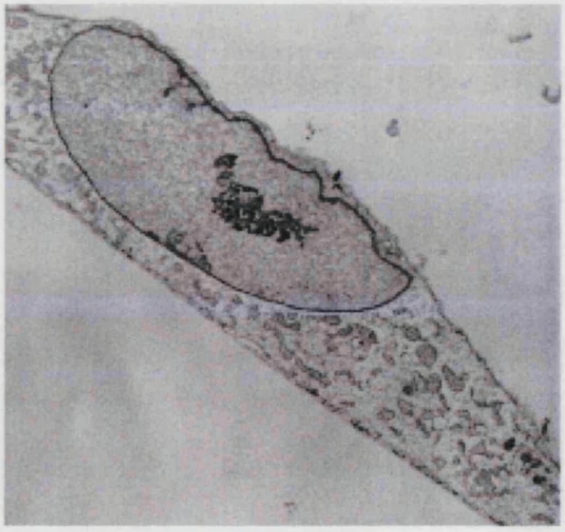


Figure 8(a) This EM photo shows healthy cells with intact membranes, dispersed chromatin and numerous healthy mitochondria (Total magnification x 5,700). (b) shows an area of muscle fibres with an arrow indicating an intercalated disc (Total magnification x 5,700).



(c) This electron micrograph shows a fibroblast. Although pre-plating removed the vast majority of fibroblasts, a few were occasionally seen in the myocyte cultures (Total magnification x 5,700).

3.4.2 A Model of Apoptosis in Primary Cardiomyocytes

Staurosporine (SSP) is a well-known inducer of apoptosis, which functions through the inhibition of protein kinases. SSP applied at a concentration range of 0.5-20μM over 20hr produced a significant drop in cell metabolism and a corresponding release of lactate dehydrogenase (LDH) through compromised plasma membranes (Figure 9a & b). EM analysis showed that the vast majority of the cells were dying by apoptosis as pictured in Figure 10. However, from 10μM an increasing amount of necrosis was seen and at concentrations of 20μM and above necrosis was the predominant form of cell death (Figure 11). Therefore, 5μM was chosen as a working concentration.

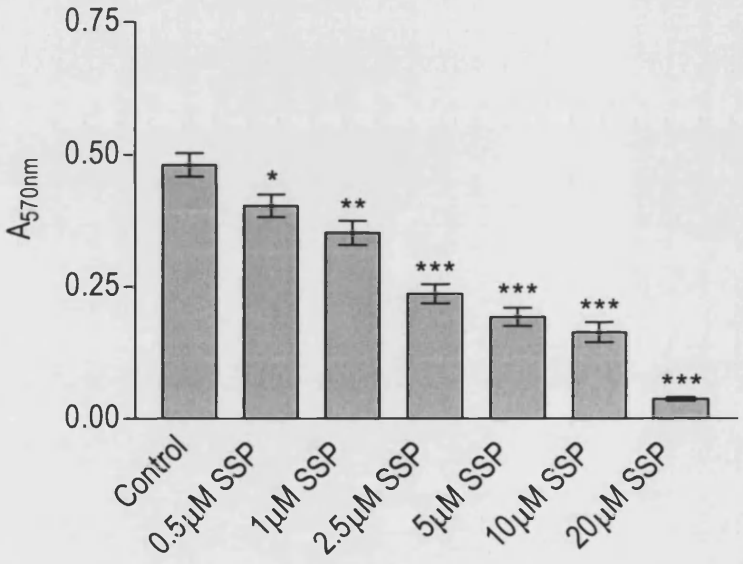


Figure 9a: Primary cardiomyocytes treated with 0.5-20μM staurosporine (SSP) for 20hr showed a significant and concentration-dependent decrease in metabolic-dependent conversion of MTT reagent [*, $p<0.05$ v. control; **, $p<0.01$ v. control; ***, $p<0.001$ v. control].

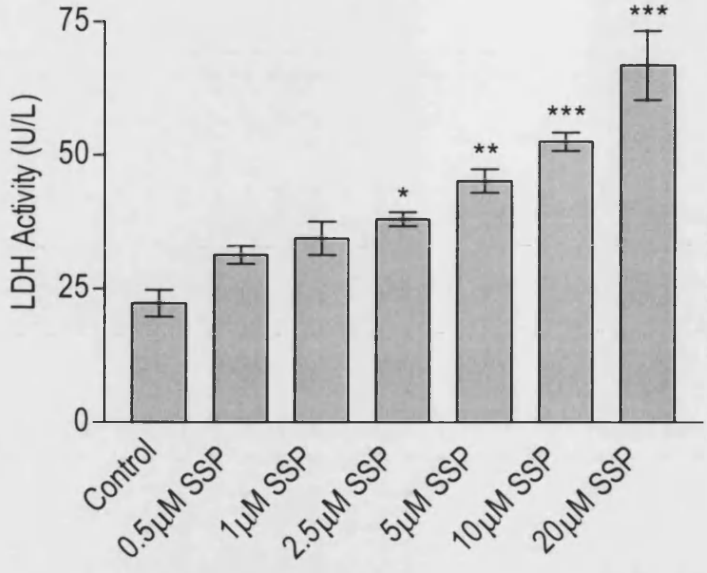


Figure 9b: Primary cardiomyocytes treated with 0.5-20μM staurosporine (SSP) for 20hr showed a significant and concentration-dependent release of LDH [*, $p<0.05$ v. control; **, $p<0.01$ v. control; ***, $p<0.001$ v. control].

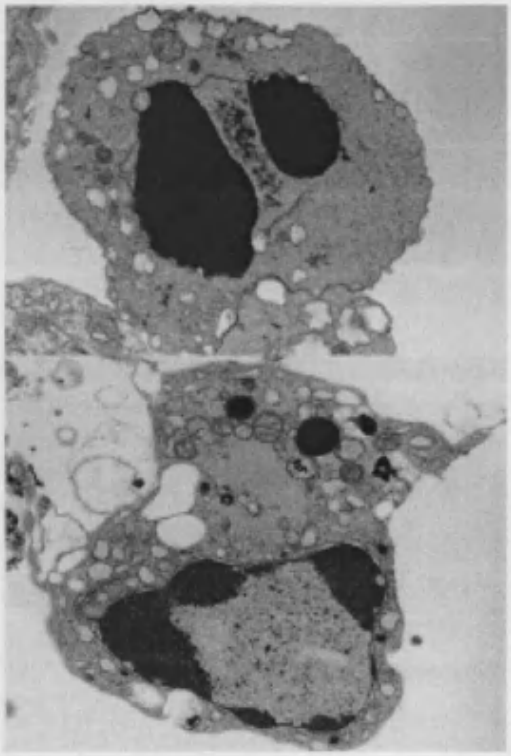


Figure 10: Electron micrograph showing primary cardiomyocytes following 20hr treatment with 5μM staurosporine (Total magnification x 5,700). These cardiomyocytes display typical signs

of apoptosis in comparison to the untreated cardiomyocytes in Figure 4a & b. The chromatin is condensed and the cell has retracted and rounded, but the cell membranes and organelles are intact.

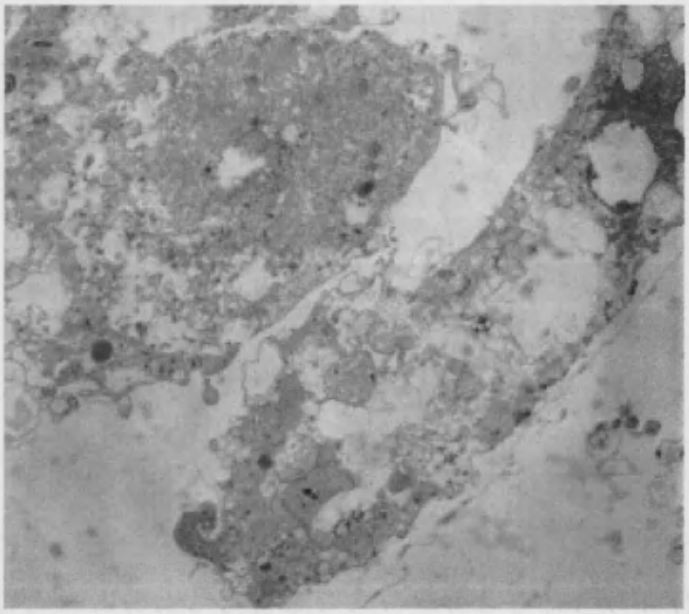


Figure 11: This electron micrograph shows primary cardiomyocytes typical of those that were treated for 24hr with 20μM staurosporine (Total magnification x 5,700). The cardiomyocytes are displaying symptoms characteristic of necrosis with broken cell membranes and disruption so extreme that the nuclei are not obvious. The vacuoles are enlarged and the lysosomes and mitochondria are disrupted.

In this study, several methods were utilised for the quantification of apoptotic cardiomyocytes, as currently there is no definitive single available method suitable for the measurement of apoptosis. Measurement of cellular metabolism is inappropriate as high metabolic activity is required for implementation of apoptosis and results will therefore be deceptive. The release of enzymes from cells with compromised plasma membranes is a widely used method, but since apoptotic cells retain plasma membrane integrity the assay can only measure post-apoptotic necrotic changes. Analysis of PI-stained cells by flow cytometry as described in Chapter 2

distinguishes apoptotic cells through a combination of decreased staining due to DNA fragmentation and decrease in cell size. This is therefore a direct assay of cell death but could potentially also detect necrotic cells with fragmented DNA as well as apoptotic ones and would consequently be unreliable for distinguishing between apoptosis and necrosis. This problem also applies to other popular methods such as Hoescht and TUNEL staining. Additionally there is the problem of whether the cell death occurring is 'true' apoptosis, necrosis or a combination of both processes.

To address this problem, 3 assays were used in conjunction to measure the viability of primary cardiomyocytes during 24hr of 5μM SSP application. Cellular metabolism as assayed by conversion of MTT to MTT formazan displayed a decreasing trend over 18 hours but only became significantly reduced by 24 hours (Figure 12a). These results were confirmed by measurement of the release of LDH from cells with compromised plasma membranes (Figure 12b). However analysis of SSP-treated cardiomyocytes by flow cytometry using a combination of cell size and DNA staining showed a significant decrease in cell viability after only 4hr. Prior to DNA staining with propidium iodide (PI), the cells were permeablised to allow diffusion of small DNA fragments so that cells with fragmented DNA would be detectable by their reduced DNA content shown as a decreased fluorescence. Fluorescence of PI was plotted against cell size as measured by forward scatter and the results presented as a dot plot (Figure 13a). This clearly showed 2 groups of cells, 1 group of poorly stained, reduced size dead or dying cells and 1 group of brightly stained, normal size group of viable cells. All control groups contained some dead cells, the number of which varied between primary culture preparations. Treatment with SSP caused an increase in the number of dead cells and corresponding decrease in the viable population. The decrease of viable cardiomyocytes was expressed as a percentage of the control and is shown in Figure 13b.

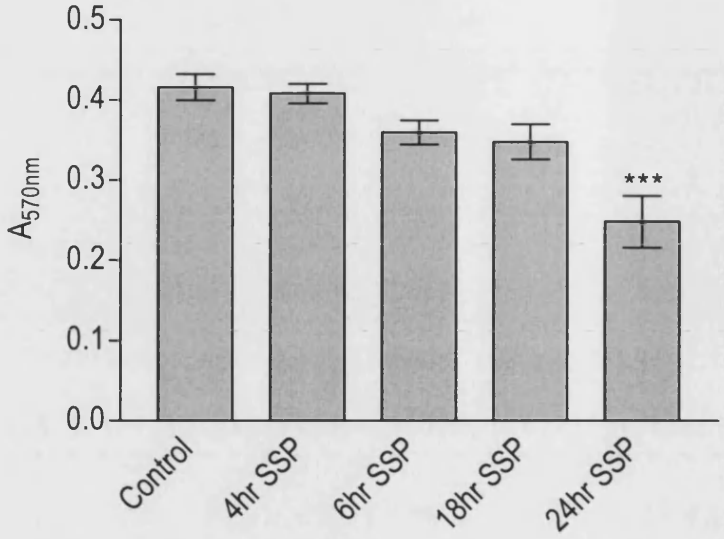


Figure 12a: primary cardiomyocytes were treated with 5μM staurosporine (SSP) for up to 24hr. Metabolic activity was then assayed by the reduction of MTT reagent over 30min, revealing a decline in activity over the duration of the treatment that became significant between 18 and 24hr [***, $p<0.001$ v. control].

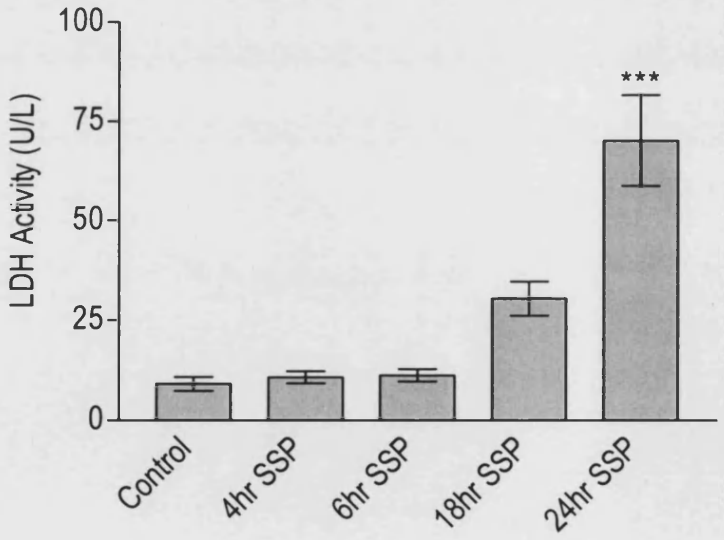


Figure 12b: prior to application of MTT reagent (fig 12a) an aliquot of culture media was reserved for analysis of LDH activity as shown in the graph above. This revealed an increased

release of LDH into the extracellular medium that attained significance between 18 and 24hr [***, p<0.001 v control].

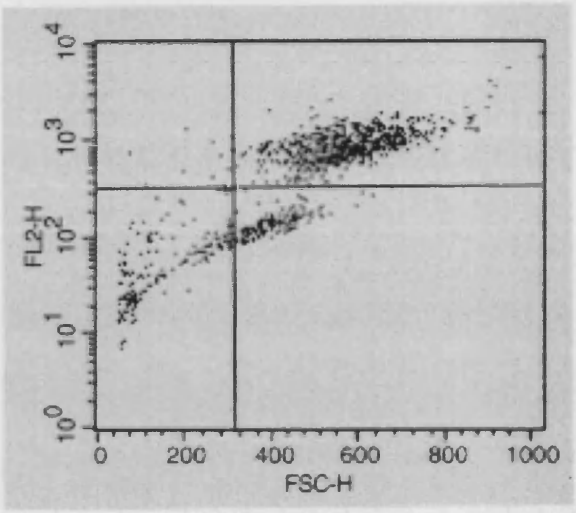


Figure 13a: Cell size (FSC-H) and propidium iodide fluorescence (FL2-H) were the measured by flow cytometry and plotted in a scatter plot, an example of which is shown above. This form of analysis revealed a distinct group of cell small poorly stained cells allowing straightforward quantification of the number of large cells with noteworthy staining of DNA (and therefore viable) in the upper-right quadrant.

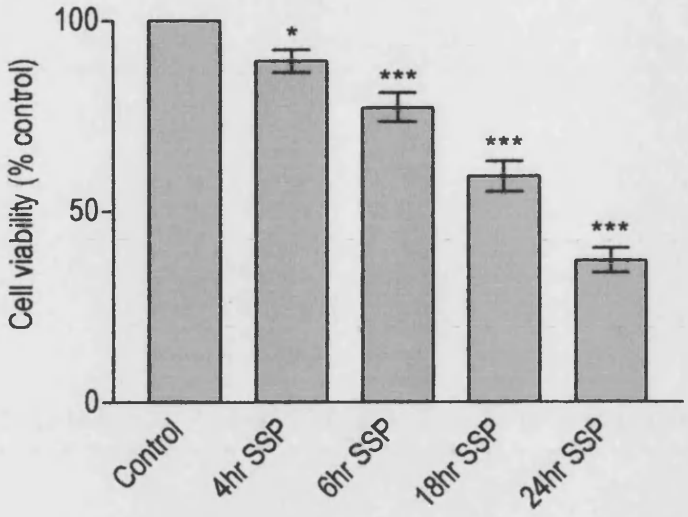


Figure 13b: the number of viable cells in the upper-right quadrant of the dot plot (shown in figure 13a) was expressed as a percentage of the control. This showed a significant decrease in the number of viable cells over 24hr of 5μM staurosporine (SSP) treatment [*, $p < 0.05$; ***, $p < 0.001$ v. control].

In addition to these quantitative assays, it was noted that treatment with SSP caused cleavage of the 32kD caspase 3 precursor as illustrated in figure 14. Lysates of untreated control cardiomyocytes were probed with an antibody to the caspase 3 precursor by Western blotting. This revealed a clear band of protein at 32kD. However, when lysates of cardiomyocytes that had been treated with SSP for 24hr were probed with the same antibody, no protein bands were observed, indicating cleavage of the inactive precursor of caspase 3 into the active form. Activity of the caspase family of proteins has many times been detected during apoptotic cell death and activation of the caspases, especially caspase 3, has often been used as evidence of cell death proceeding by apoptotic pathways.

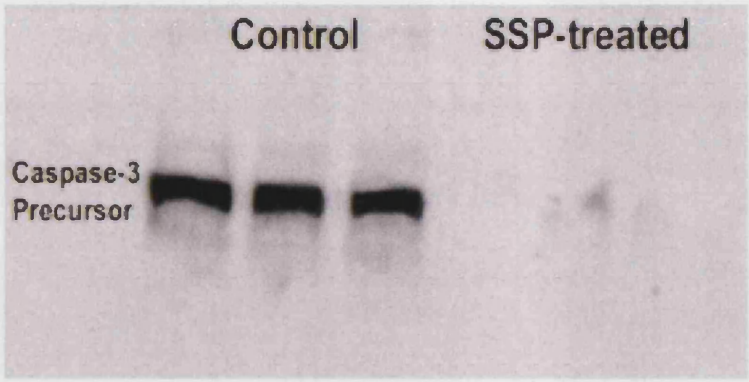


Figure 14: A representative Western blot showing that treatment of primary cardiomyocytes with 5μM staurosporine for 24hr resulted in cleavage of the caspase 3 precursor protein that was not seen in control cells.

3.4.3 A Model of Necrosis in Primary Cardiomyocytes

T-butylhydroperoxide (t-BOOH) has been frequently used for the induction of oxidative stress and cell death in a wide variety of cell types. Application of 60-140μM t-BOOH for 1hr caused a significant drop in MTT conversion by primary cardiomyocytes and a corresponding increase in LDH release (Figure 15a & b). Analysis by electron microscopy confirmed that the cells were undergoing necrotic cell death as shown in Figure 15c.

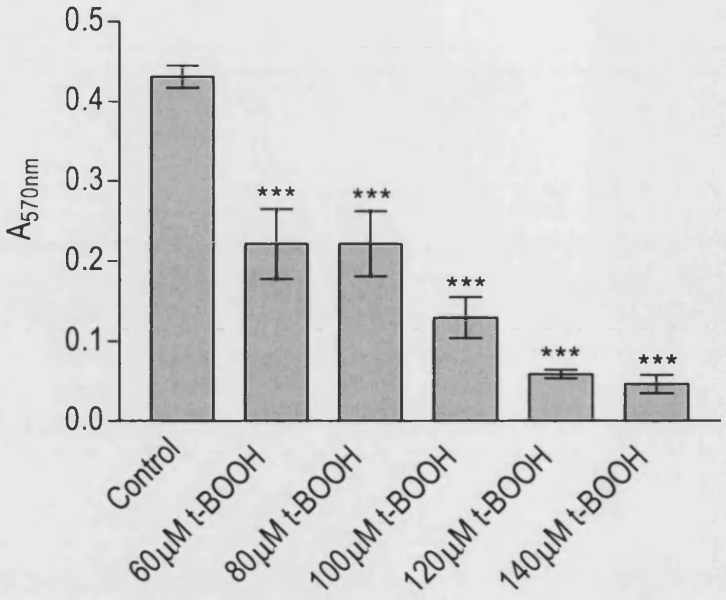


Figure 15a: 1hr of treatment with 60-140μM t-butylhydroperoxide (t-BOOH) produced a concentration dependent decrease in cell metabolism as measured by conversion of MTT reagent [***, $p < 0.001$ v. control].

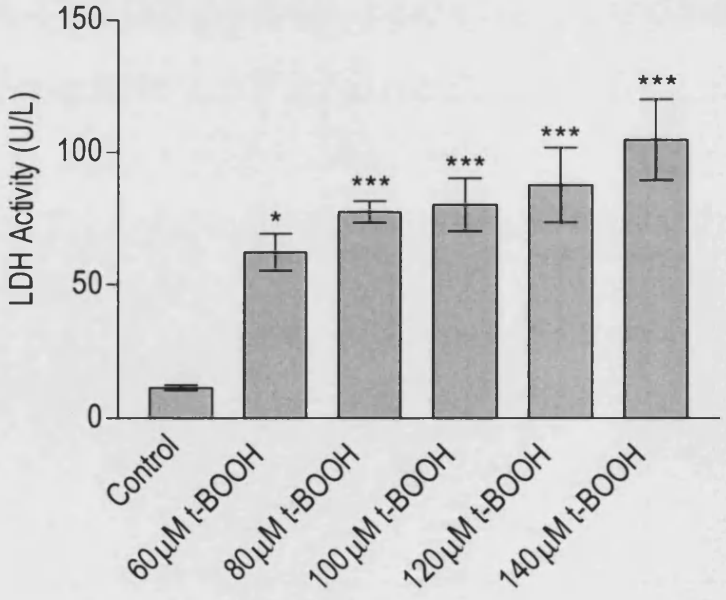


Figure 15b: the same cells assayed for release of LDH displayed a concentration dependent

increase, showing loss of membrane integrity as a consequence of t-butylhydroperoxide (t-BBOH) treatment [*, $p < 0.05$; ***, $p < 0.001$ v. control].

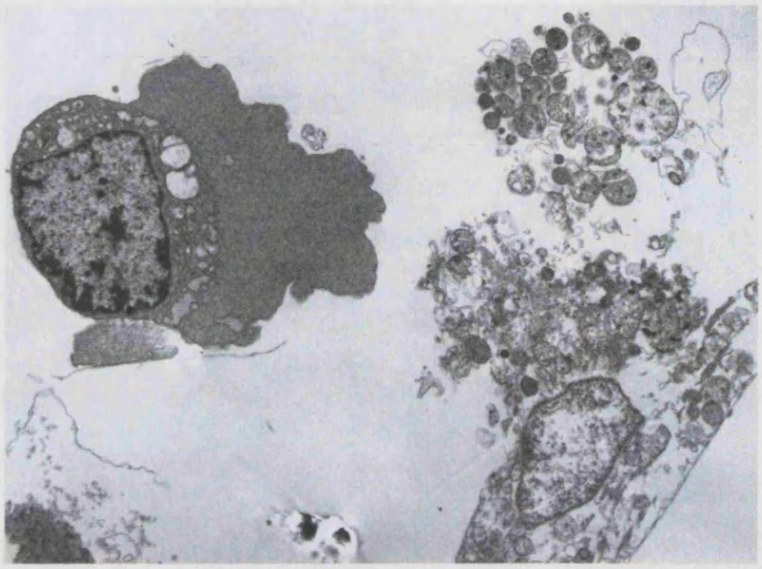


Figure 15c: Primary cardiomyocytes following application of 100μM t-butylhydroperoxide for 1hr. The cell membranes have broken open and the contents spilled out. The cell organelles have suffered only minimal disruption, probably reflecting the short treatment time (Total magnification x 5,700).

3.4.4 A Model of Simulated Ischaemia-Reperfusion Injury

Primary cardiomyocytes were subjected to treatment with ischaemic solution for up to 6 hours as described in Materials and Methods followed by simulated reperfusion consisting of incubation for up to 24hr in full maintenance media. Analysis of the primary cardiomyocytes by electron microscopy revealed that the predominant form of cell death was necrosis as shown in Figure 16i & j. Measurement of cellular metabolism by MTT showed a steady decline in metabolic activity during the selected time periods as shown. LDH release followed a similar trend except in the case of simulated ischaemia with no reperfusion in which case the LDH

release was extremely low (Figure 16a – h). As this is a direct contradiction of the MTT results and the LDH release was actually consistently significantly lower than the control, it is probable that some element of the ischaemic solution used inhibits LDH activity - the most likely candidate being the 20mM lactate. This also leads to the possibility that the increased LDH activity over reperfusion time could in fact be the effect of the solution wearing off rather than increased LDH release from the cells. However, this scenario can probably be discounted since the LDH release correlates with the death of the cells as measured by MTT conversion. Simulated reperfusion of cardiomyocytes following application of ischaemic solution resulted in a gradual decline in viability up to 18hr reperfusion followed by a sharp decrease between 18 and 24hr. 3hr of simulated ischaemia with 18hr simulated reperfusion was selected for working experimental conditions. This produced a reduction in MTT conversion of approximately 50% and was therefore considered an appropriate level of cell death for the application of inhibitors. Additionally, an 18hr period of simulated reperfusion preceded the sharp decline that would suggest the sudden and irreversible death of the majority of the cells.

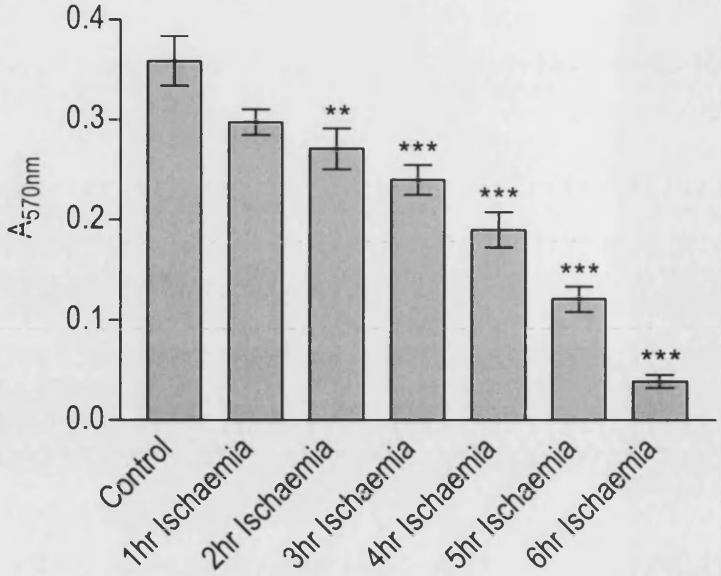


Figure 16a: Metabolic activity of primary cardiomyocytes as measured by conversion of MTT reagent, showed a time-dependent decrease during 6hr application of the simulated ischaemia solution [**, $p < 0.01$ v. control; ***, $p < 0.001$ v. control].

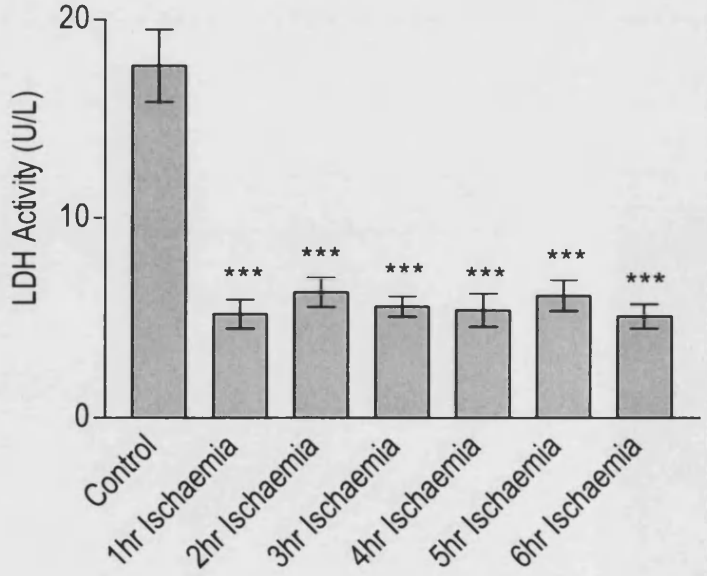


Figure 16b: LDH activity during treatment with the simulated ischaemia solution was significantly lower than control suggesting that LDH activity was severely inhibited by the solution used [***, $p < 0.001$ v. control].

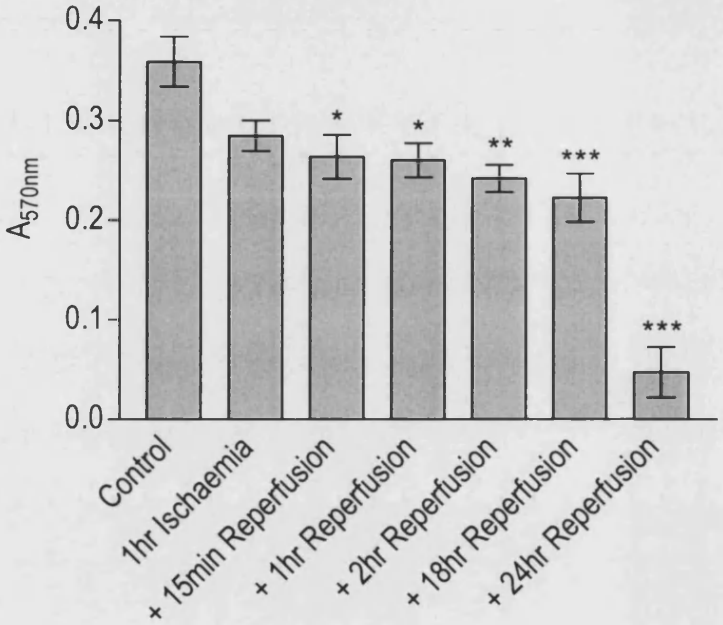


Figure 16c: A further time-dependent decrease in cardiomyocyte metabolic activity was seen during 24hr of simulated reperfusion following 1hr of treatment with the simulated ischaemia solution. A slight decrease was observed during the first 18hr with a sharp decline from 18 to 24hr [*, $p < 0.05$ v. Control; **, $p < 0.01$ v. Control; ***, $p < 0.001$ v. Control].

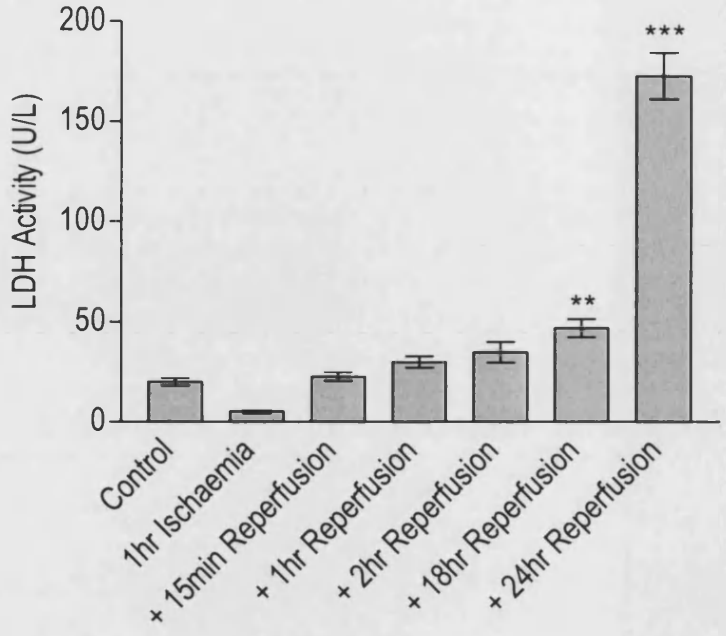


Figure 16d: A time-dependent increase in LDH release was observed during 24hr of simulated reperfusion following 1hr of treatment with the simulated ischaemia solution. A slight increase was seen during the first 18hr with a sharply increased release evident from 18 to 24hr [*, $p < 0.05$ v. Control; ***, $p < 0.001$ v. Control].

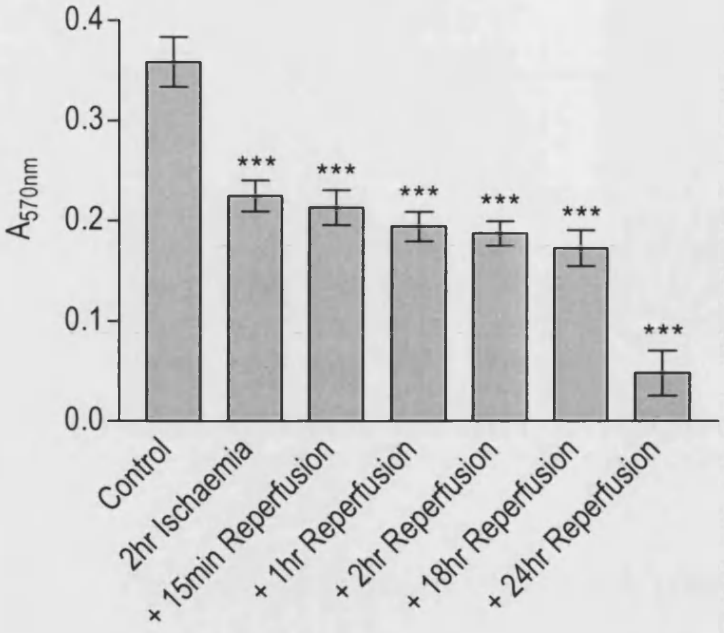


Figure 16e: A further time-dependent decrease in cardiomyocyte metabolic activity was seen during 24hr of simulated reperfusion following 2hr of treatment with the simulated ischaemia solution. A slight decrease was observed during the first 18hr with a sharp decline from 18 to 24hr [***, $p < 0.001$ v. Control].

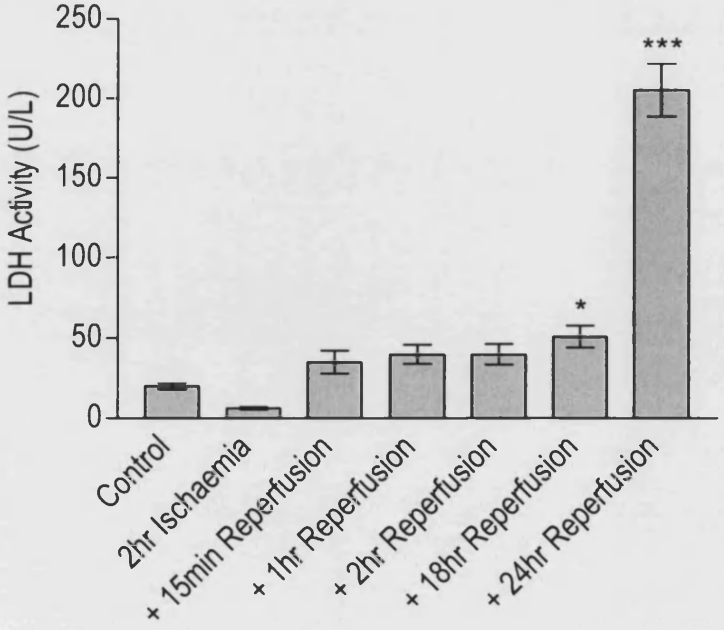


Figure 16f: A time-dependent increase in LDH release was observed during 24hr of simulated reperfusion following 2hr of treatment with the simulated ischaemia solution. A slight increase was seen during the first 18hr with a sharply increased release evident from 18 to 24hr [*, $p < 0.05$ v. Control; ***, $p < 0.001$ v. Control].

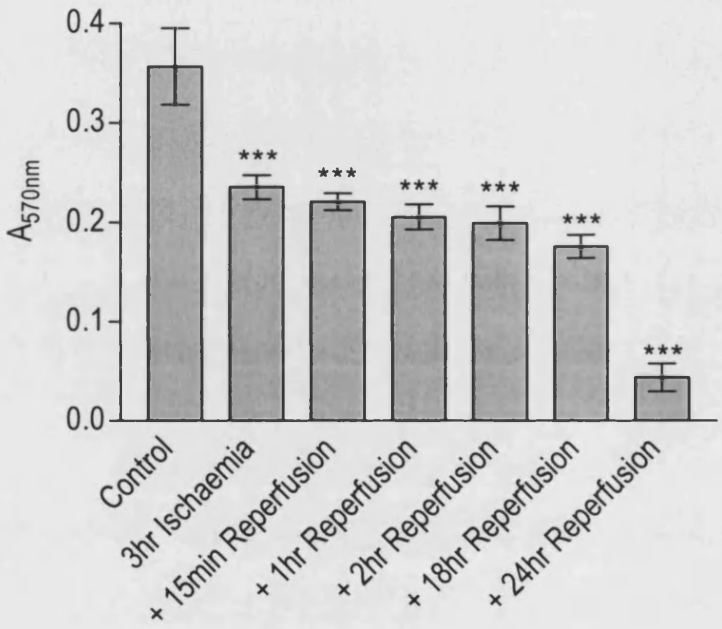


Figure 16g: A further time-dependent decrease in cardiomyocyte metabolic activity was seen during 24hr of simulated reperfusion following 3hr of treatment with the simulated ischaemia solution. Significant reduction of activity was observed during the first 18hr of simulated reperfusion, with a further sharp decline over the final 6 hours [***, $p < 0.001$ v. Control].

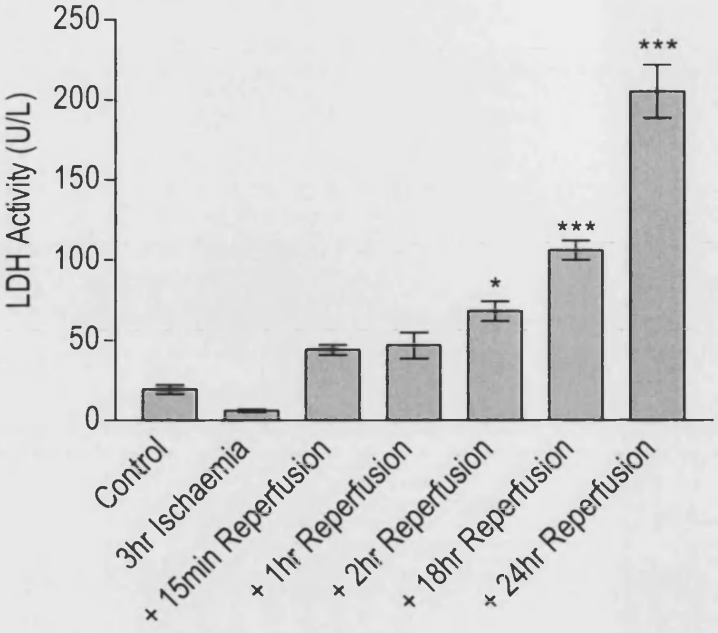


Figure 16h: Significant, time-dependent release of LDH from primary cardiomyocytes was seen during simulated reperfusion following 3hr of treatment with the simulated ischaemia solution. Significant release of LDH was observed during the first 18hr of simulated reperfusion, with a further sharp increase over the final 6 hours [*, $p < 0.05$ v. control; ***, $p < 0.001$ v. control].

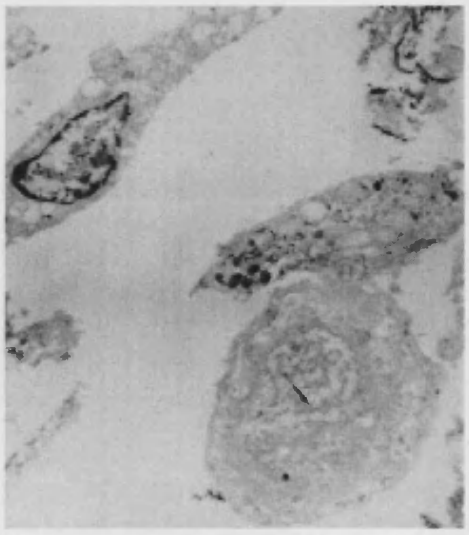


Figure 16i: primary cardiomyocytes subjected to 3hr application of simulated ischaemia solution displayed characteristics of necrosis. The cells in this EM photograph have

marginalized chromatin, disrupted mitochondria and distended ER. Some cells were observed to have intact plasma membranes whereas other cells had burst open (Total magnification x 5,700).

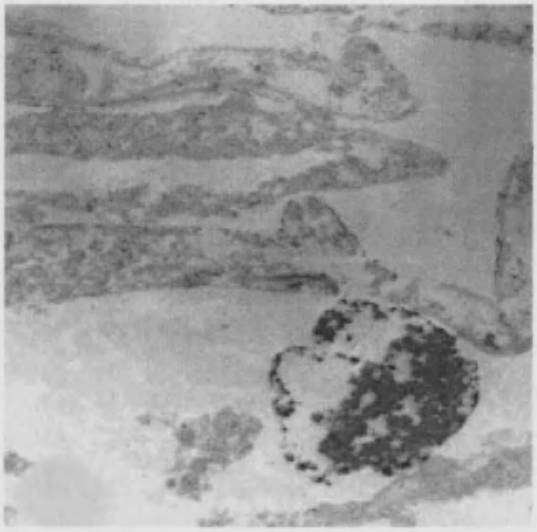


Figure 16j: 24hr simulated reperfusion (following 3hr simulated ischaemia solution treatment) killed the majority of the cells, also by necrotic death. The cells in this image show completely irreversible damage. They have disintegrated and unrecognisable organelles, the chromatin has also disintegrated and the plasma membranes are broken in places (Total magnification x 5,700).

3.4.5 The Effect of MPTP Inhibition on Apoptosis

CsA was applied to the primary cardiomyocytes for 2hr at a concentration range of 10nM-1μM. Analysis of MTT conversion and LDH activity both revealed that CsA became toxic between 400 and 600nM (Figure 17a & b).

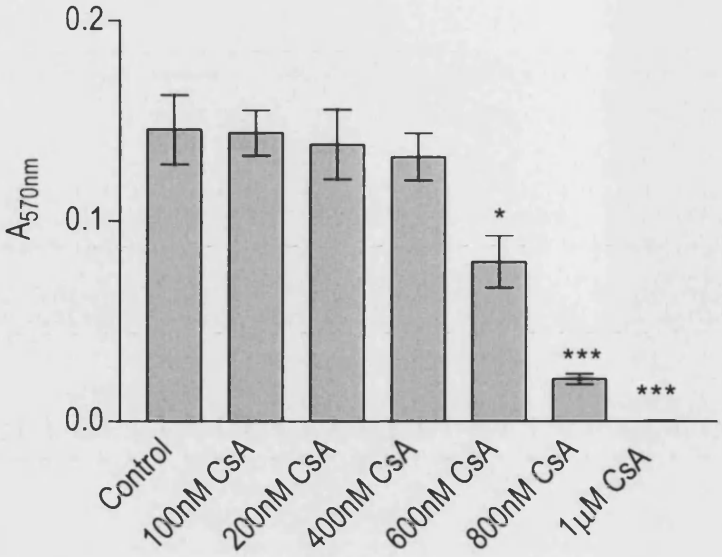


Figure 17a: Primary cardiomyocytes were treated with cyclosporin A (CsA) 100nM-1μM for 2hr. Analysis of metabolic activity by conversion of MTT reagent showed that CsA attained toxicity between 400 and 600nM [*, $p<0.05$; ***, $p<0.001$ v control].

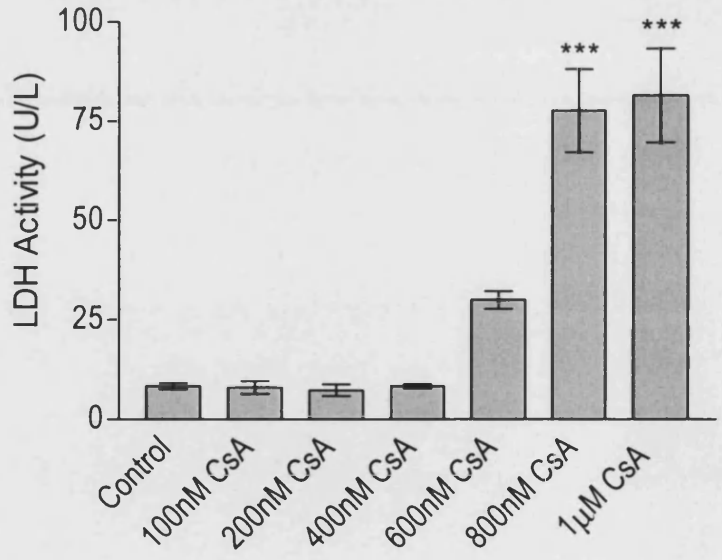


Figure 17b: Prior to MTT analysis, an aliquot of culture media was removed for analysis of LDH activity. This showed that cyclosporin A (CsA) became significantly toxic between 600 and

800nM although there was a notable increase in LDH release between 400 and 600nM [***, $p < 0.001$ v control].

CsA was applied to the cardiomyocytes for 1hr in a range of non-toxic concentrations and then washed off prior to 20hr of SSP treatment. However, analysis of both metabolism and LDH release showed that the application of 100-400nM CsA followed by 5μM SSP for 20hr was in fact more toxic to the primary cardiomyocytes than the SSP alone (see Figure 18a & b).

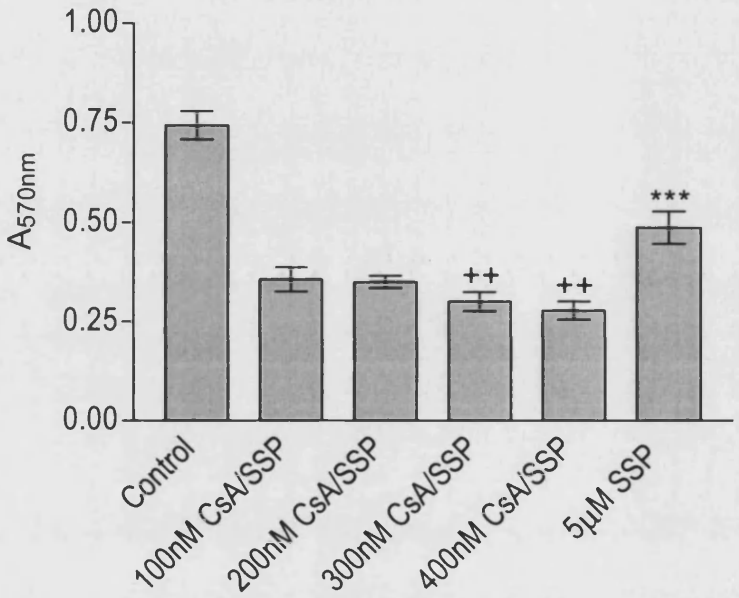


Figure 18a: Primary cardiomyocytes were treated with cyclosporin A (CsA) at concentrations ranging from 100-400nM followed by 5μM staurosporine (SSP) for 20hr. Assessment of metabolic activity showed that pre-treatment with CsA led to a further reduction in of activity compared to SSP alone [***, $p < 0.001$ v control; ++, $p < 0.01$ v 5μM SSP].

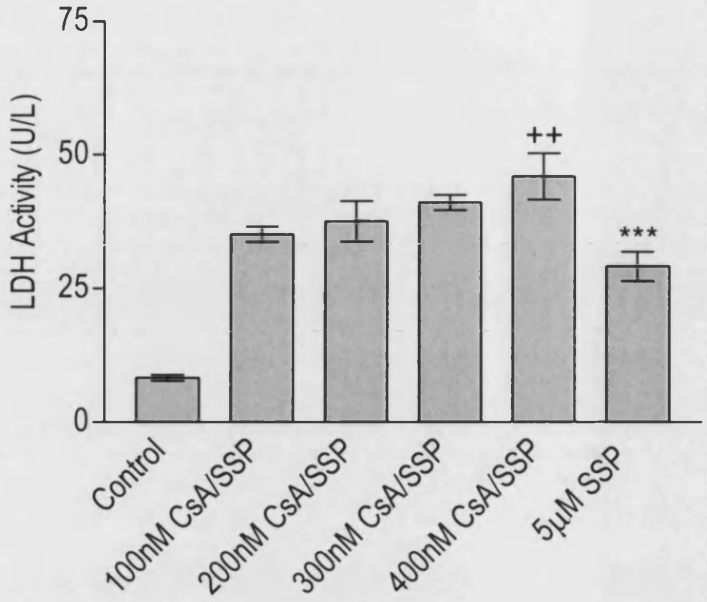


Figure 18b: The same cells assayed for LDH release showed that all concentrations of cyclosporin A (CsA) used caused an increased loss of membrane integrity compared to staurosporine (SSP) [***, $p < 0.001$ v control; ++, $p < 0.01$ v 5μM SSP].

To determine whether the length of treatment was instrumental in the toxicity of the CsA, it was applied to primary cardiomyocytes for 1hr at the same concentration range. The cells were then washed and fresh media was replaced for 20hr. Analysis of MTT conversion and LDH release after this time period showed no evidence of toxicity (Figure 19a & b).

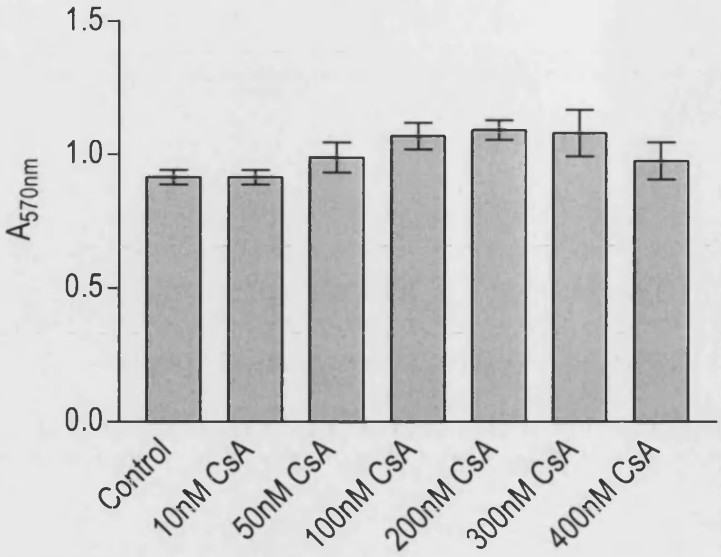


Figure 19a: Cardiomyocytes were treated with cyclosporin A (CsA) at concentrations of 100-400nM for 1hr and then reperfused for 20hr. Analysis of MTT conversion showed no significant variation in metabolic activity compared to the control.

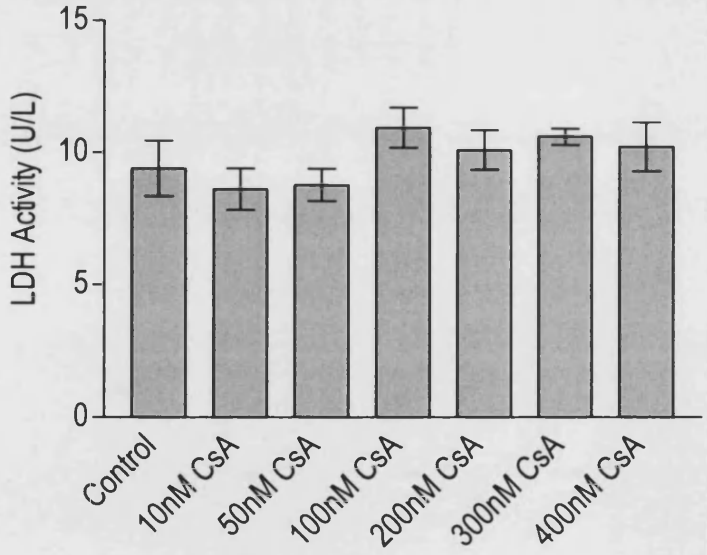


Figure 19b: Assessment of LDH activity in the same cells showed no additional loss of membrane integrity in the cyclosporin A (CsA) treated cardiomyocytes.

Therefore CsA was tested at a lower range of concentrations. Cardiomyocytes were treated with 1-100nM CsA, which was washed off after 1hr and replaced with 5µM SSP. Although there was no evidence of CsA toxicity below 80nM, no indication of protection against SSP-induced apoptosis could be seen by either MTT (Figure 20a), LDH assay (Figure 20b) or by assessment of DNA content in permeablised cells (Figure 20c).

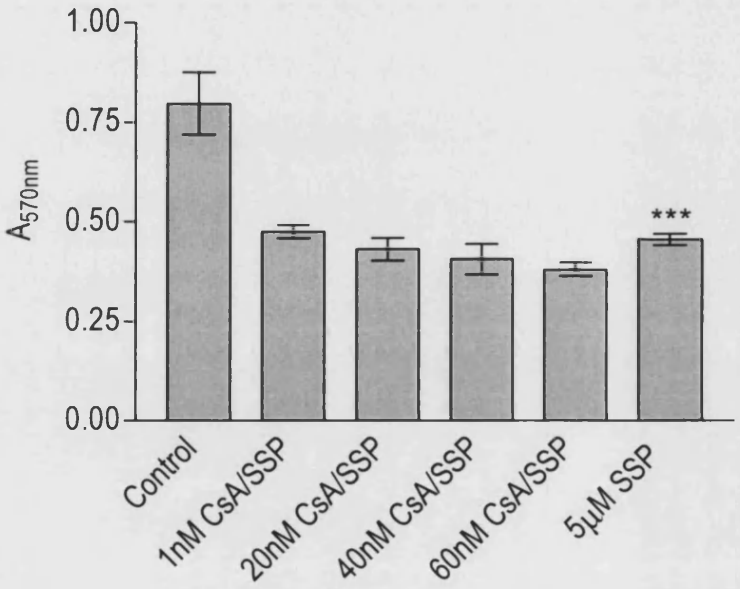


Figure 20a: Primary cardiomyocytes were treated with cyclosporin A (CsA) at concentrations ranging from 1-60nM followed by 5µM staurosporine (SSP) for 20hr. The reduction of metabolic activity (conversion of MTT reagent) proved to be unaffected by CsA pre-treatment [***, $p < 0.001$ v control].

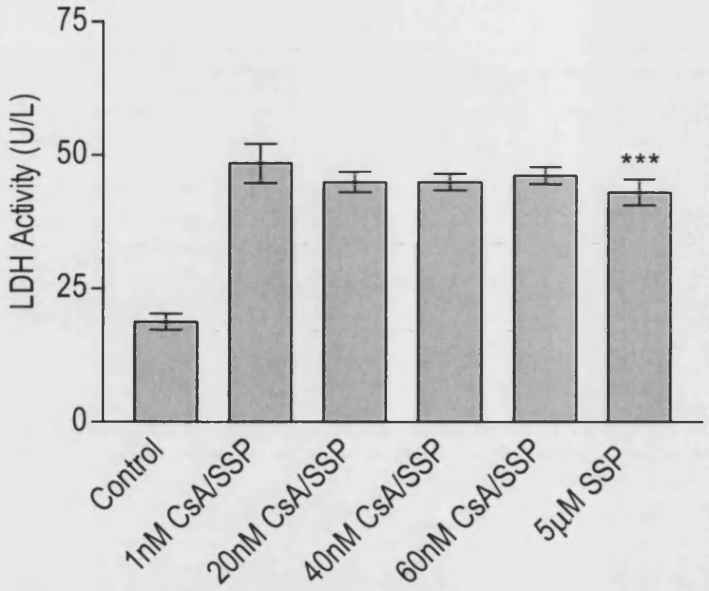


Figure 20b: The same cardiomyocytes assayed for LDH release showed that pre-treatment with cyclosporin A (CsA) at 1-60nM did not inhibit the staurosporine (SSP)-induced loss of membrane integrity [***, $p < 0.001$ v. control].

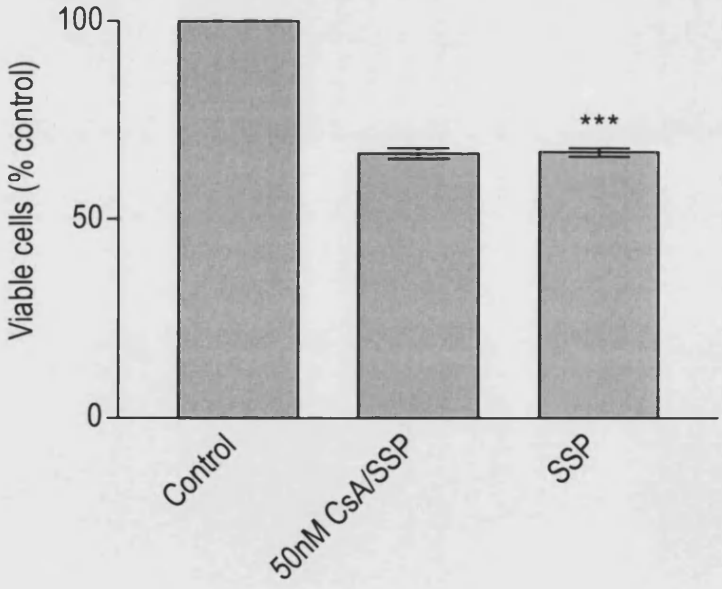


Figure 20c: the number of viable cells according to size and DNA content was expressed as a percentage of the control. This showed that 50nM cyclosporin A (CsA) was unable to inhibit staurosporine (SSP)-induced loss of cell viability [***, $p<0.001$ v. control].

Primary cardiomyocytes were treated for 1hr with CsA and 5μM SSP concurrently. Measurement of metabolic activity and membrane integrity after 20hr showed that the combination of CsA and SSP was significantly toxic to the cardiomyocytes (Figure 21a & b).

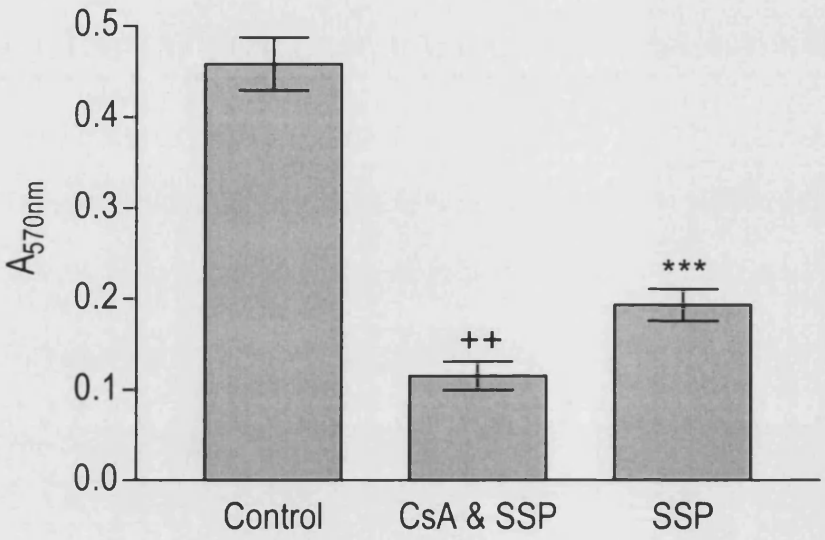


Figure 21a: Primary cardiomyocytes were treated with 50nM cyclosporin A (CsA) concurrently with 5μM staurosporine (SSP) for 1hr. The combination of CsA and SSP induced a significantly greater reduction of metabolic activity (quantified by conversion of MTT reagent) than SSP alone [***, $p < 0.001$ v. control; ++, $p < 0.01$ v. SSP].

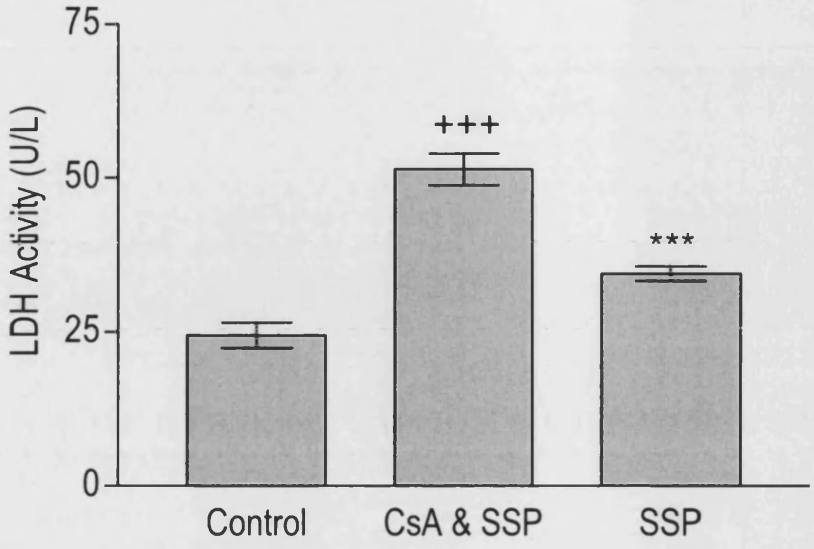


Figure 21b: The same cardiomyocytes assayed for LDH release showed that the combination of cyclosporin A (CsA) and staurosporine (SSP) induced a significantly greater release of LDH than SSP alone [***, $p < 0.001$ v. control; +++, $p < 0.001$ v. SSP].

1-25μM Bongkrekic acid (BA) (Figure 22) was applied to primary cardiomyocytes for 2hr. Analysis of MTT conversion and LDH release after this time showed a significant toxic effect from between 10 and 15μM (Figure 23a & b).

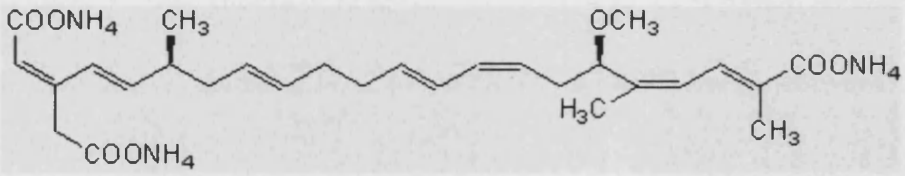


Figure 22: Structure of bongkrekic acid (molecular formula: $C_{28}H_{38}O_7$)

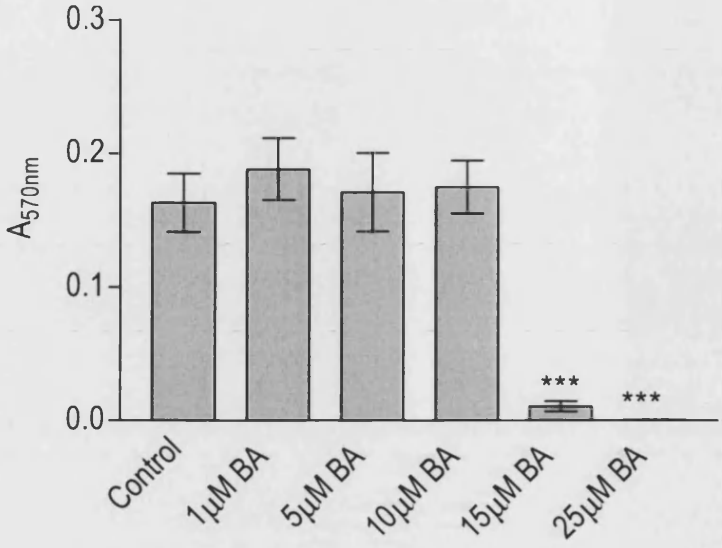


Figure 23a: Primary cardiomyocytes treated with bongkrekic acid (BA) for 2hr showed a significant decrease in metabolic activity (quantified by conversion of MTT reagent) between 10 and 15μM [***, $p < 0.001$ v. control].

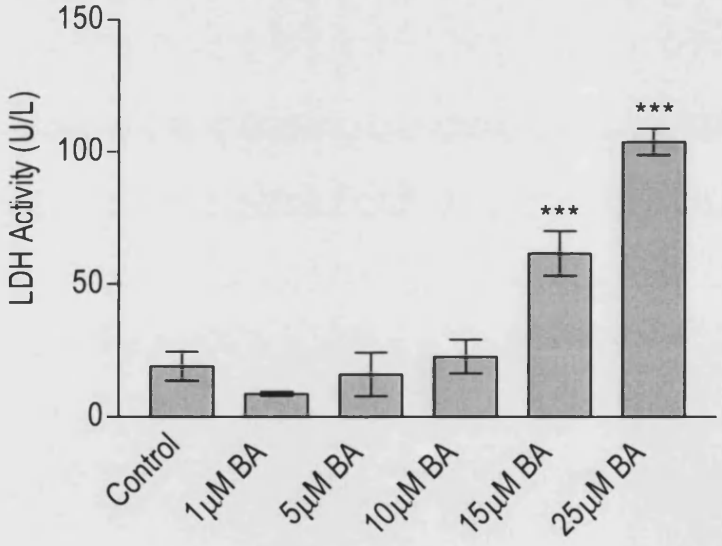


Figure 23b: the same cardiomyocytes assayed for LDH release showed a significant loss of membrane integrity between 10 and 15μM bongkrekic acid (BA) [***, $p < 0.001$ v. control].

BA was applied to primary cardiomyocytes for 1hr at a concentration range of 1-10μM. The cells were then washed with PBS and 5μM SSP was applied for 20hr. Analysis of metabolic activity and membrane integrity showed that BA was unable to inhibit apoptosis as shown in figure 24a & b.

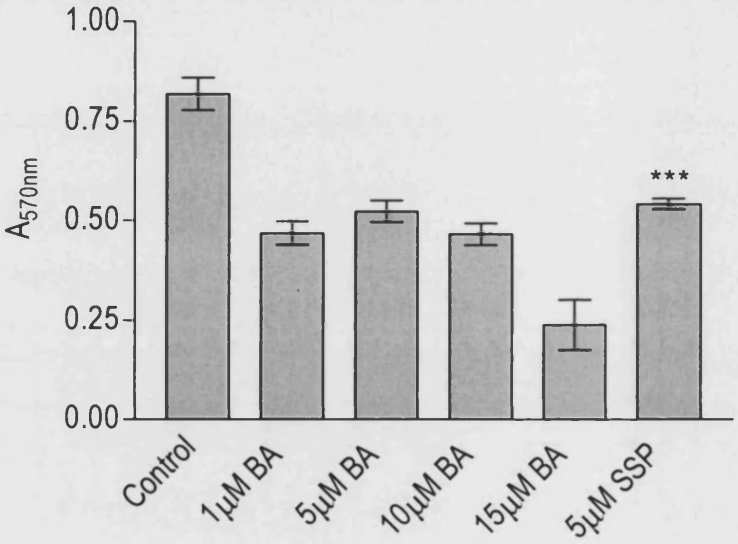


Figure 24a: Primary cardiomyocytes were treated with bongkrekic acid (BA) at concentrations ranging from 1-10μM followed by 5μM staurosporine (SSP) for 20hr. The reduction of metabolic activity (quantified by conversion of MTT reagent) proved to be uninhibited by BA pre-treatment [***, $p < 0.001$ v. control].

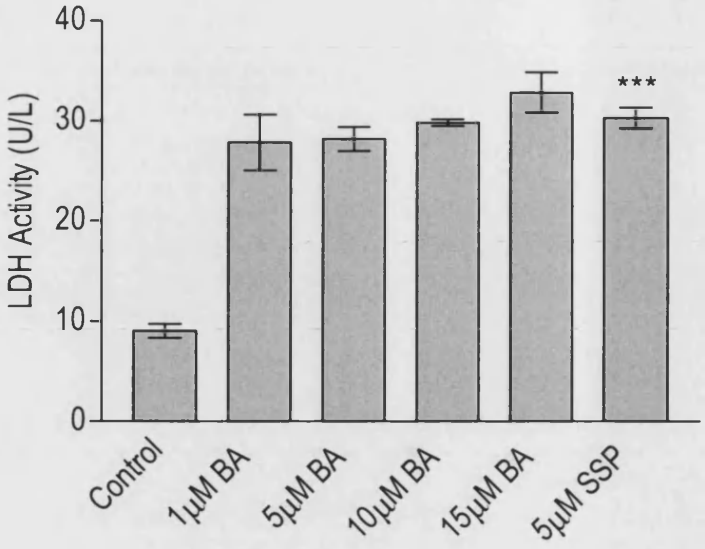


Figure 24b: The same cardiomyocytes assayed for LDH release showed that pre-treatment with bongkrekic acid (BA) at 1-10μM did not inhibit the loss of membrane integrity caused by staurosporine (SSP) [***, $p < 0.001$ v. control].

Therefore 5 & 10μM BA was applied to the cardiomyocytes concurrently with SSP. Analysis of MTT conversion and LDH release after 20hr showed that BA was unable to inhibit cell death (Figure 25a & b).

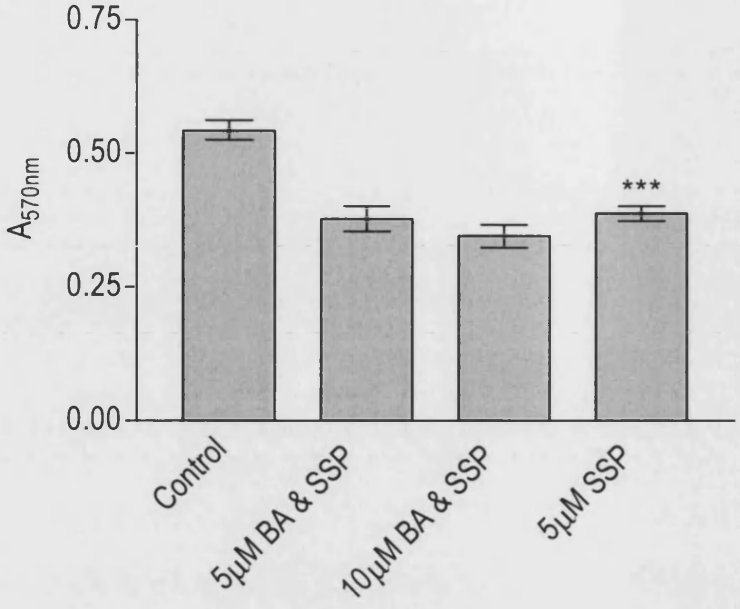


Figure 25a: Bongkrekic acid (BA) was applied concurrently with staurosporine (SSP) for 20hr. The reduction of metabolic activity (quantified by conversion of MTT reagent) was unaffected by BA [***, $p < 0.001$ v. control].

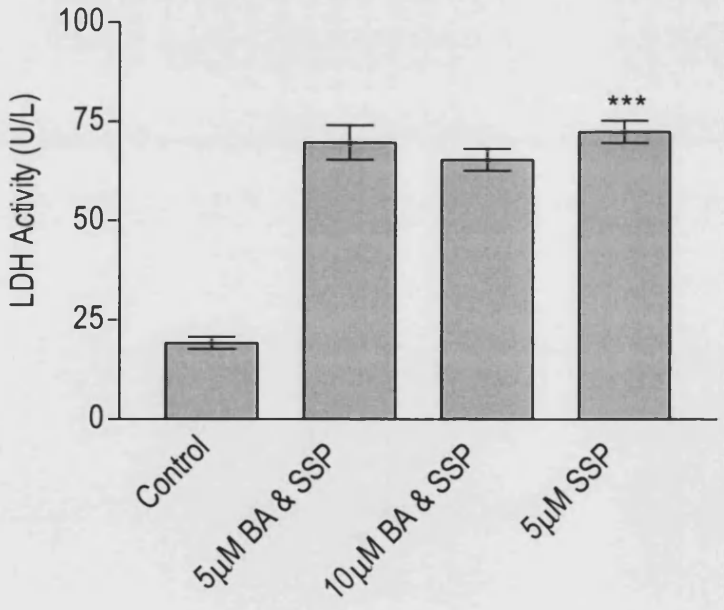


Figure 25b: The same cardiomyocytes assayed for LDH release showed that bongkrekic acid (BA) was unable to prevent the loss of membrane integrity caused by staurosporine (SSP) [***, $p < 0.001$ v. control].

3.4.6 The Effect of MPTP Inhibition on Necrosis

CsA at concentrations of 50-400nM was applied to primary cardiomyocytes for 1hr prior to treatment with 100μM t-BOOH. No inhibition of cell death could be seen by analysis of metabolic activity but 40-50nM CsA caused a slight inhibition in loss of plasma membrane integrity (Figure 26a & b).

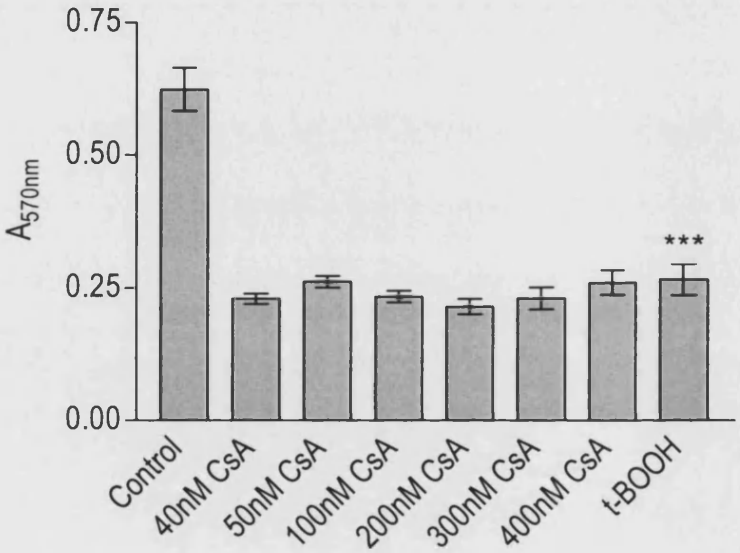


Figure 26a: Primary cardiomyocytes were treated with cyclosporin A (CsA) at concentrations ranging from 40-400nM followed by 100μM t-butylhydroperoxide (t-BOOH) for 1hr. The reduction of metabolic activity (quantified by conversion of MTT reagent) proved to be unaffected by CsA pre-treatment [***, $p < 0.001$ v. control].

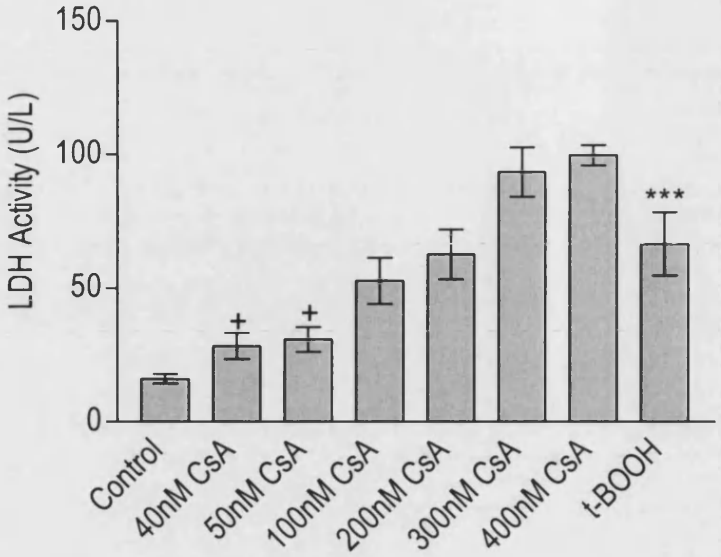


Figure 26b: The same cardiomyocytes assayed for LDH activity showed that cyclosporin A (CsA) slightly prevented loss of t-butylhydroperoxide (t-BOOH) caused membrane integrity at 40-50nM [***, $p < 0.001$ v. control; +, $p < 0.05$ v. t-BOOH].

As the effect of CsA pre-treatment remained undetermined, CsA was instead applied to the primary cardiomyocytes concurrently with t-BOOH. Analysis of metabolic activity and plasma membrane integrity showed that 40-50nM CsA significantly inhibited cell death (Figure 27a & b).

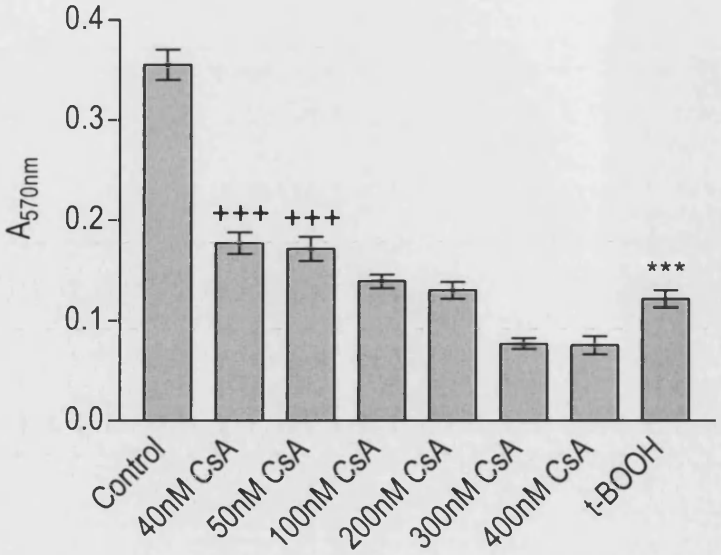


Figure 27a: Primary cardiomyocytes were treated with Cyclosporin A (CsA) at concentrations ranging from 40-400nM concurrently with 100μM t-butylhydroperoxide (t-BOOH) for 1hr. 40 & 50nM CsA significantly inhibited the reduction in metabolic activity as quantified by conversion of MTT reagent [***, $p < 0.001$ v. control; +++, $p < 0.001$ v. t-BOOH].

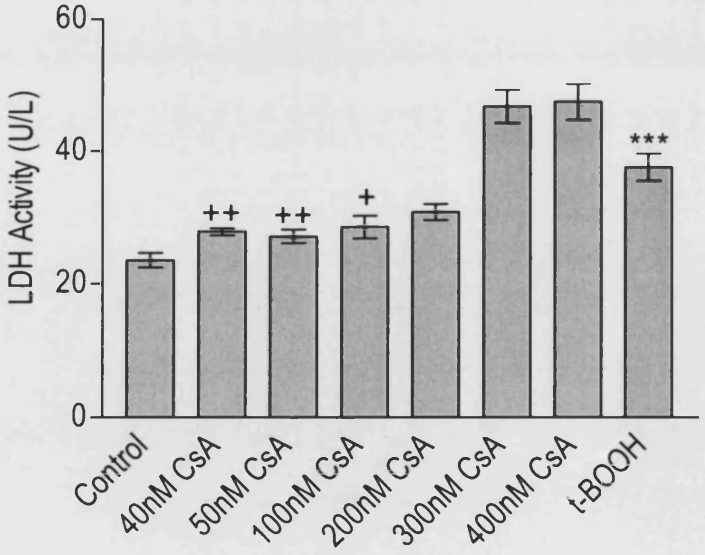


Figure 27b: The same cardiomyocytes assayed for LDH activity showed that 40-100nM cyclosporin A (CsA) significantly prevented the loss of membrane integrity caused by t-

butylhydroperoxide (t-BOOH) [***, $p < 0.001$ v. control; ++, $p < 0.01$ v. t-BOOH; +, $p < 0.05$ v. t-BOOH].

Primary cardiomyocytes were treated with 5μM BA prior to application of 100μM t-BOOH. In about half of the experiments carried out, the combination of BA and t-BOOH killed the cells and in the remainder of the experiments no inhibitory effect of BA could be seen (Figure 28a & b).

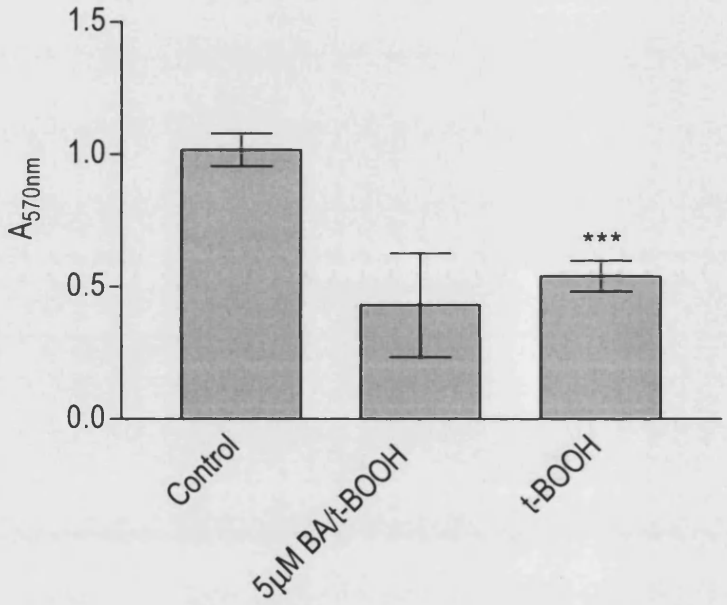


Figure 28a: Primary cardiomyocytes were treated with 5μM bongkrekic acid (BA) for 1hr followed by 100μM t-butylhydroperoxide (t-BOOH) for 1hr. The effect of BA on t-BOOH induced reduction of metabolic activity (quantified by conversion of MTT reagent) was extremely variable and inconsistent [***, $p < 0.001$ v. control].

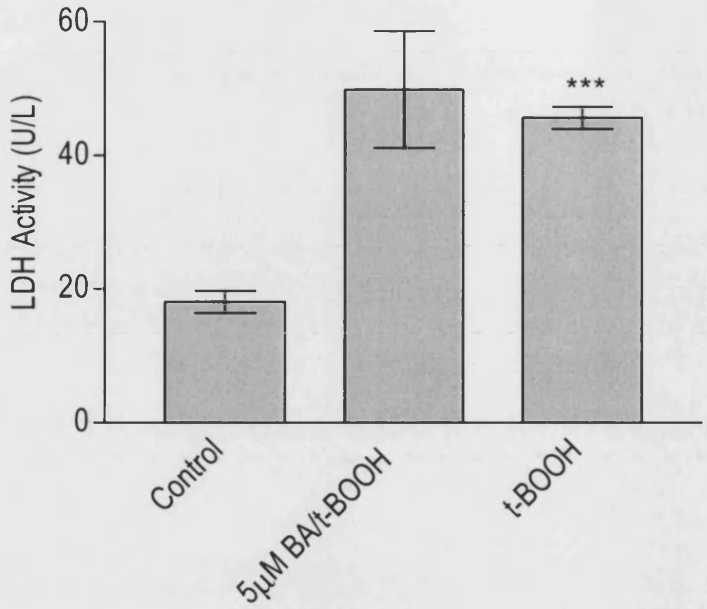


Figure 28b: The same bongkrekic acid (BA) and t-butylhydroperoxide (t-BOOH) treated cardiomyocytes assayed for LDH release showed the same inconsistency [***, $p<0.001$ v control].

Primary cardiomyocytes were then treated with BA and t-BOOH concurrently for 1hr. Analysis of both metabolic activity and plasma membrane integrity showed that the combination of BA and t-BOOH was effectively killing the cells (Figure 29a & b).

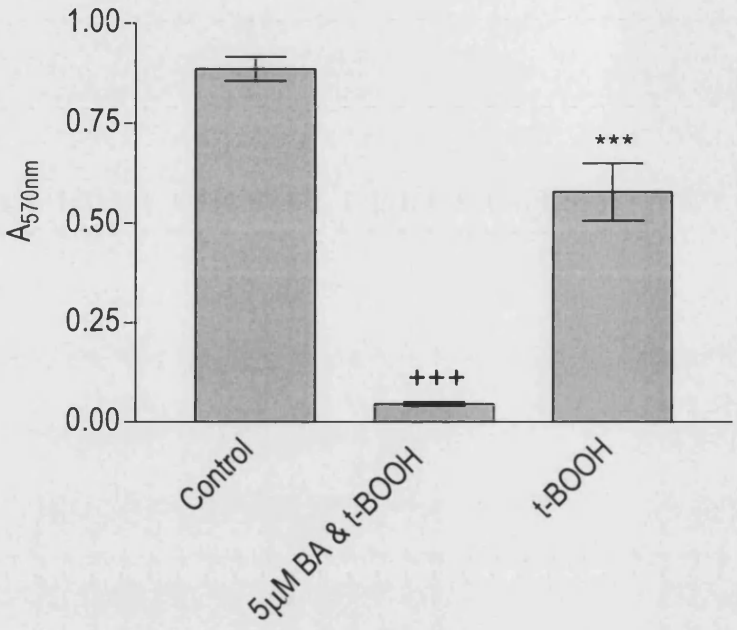


Figure 29a: Primary cardiomyocytes were treated with 5μM bongkrekic acid (BA) concurrently with 100μM t-butylhydroperoxide (t-BOOH) for 1hr. The combination of BA and t-BOOH induced a significantly greater reduction of metabolic activity (quantified by conversion of MTT reagent) than t-BOOH alone [***, $p < 0.001$ v. control; +++, $p < 0.001$ v. t-BOOH].

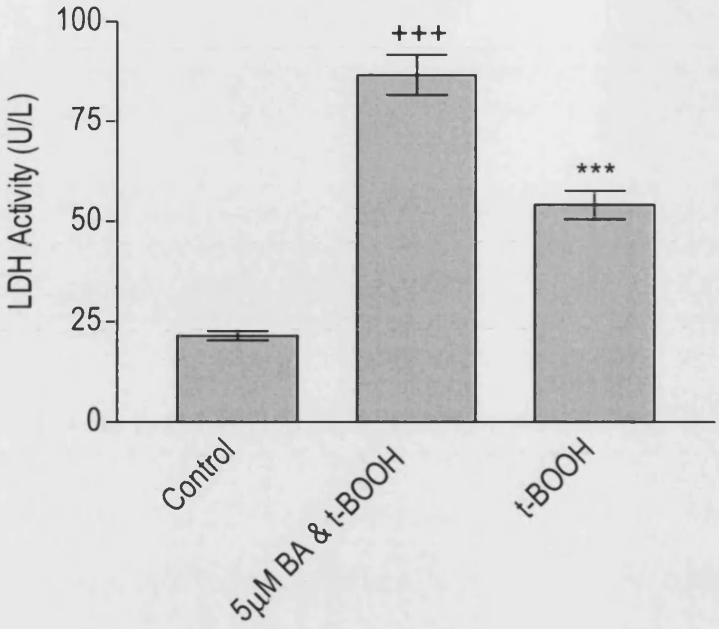


Figure 29b: The same cells assayed for LDH release showed that the combination of bongkrekic acid (BA) and t-butylhydroperoxide (t-BOOH) induced a significantly greater release of LDH than t-BOOH alone [***, $p < 0.001$ v. control; +++, $p < 0.001$ v. t-BOOH].

3.4.7 The Effect of MPTP Inhibition on Simulated Ischaemia-Reperfusion Injury

50nM CsA was applied to cardiomyocytes either, for 1hr prior to the experiment, during treatment with the simulated ischaemia solution, or during the 18hr simulated reperfusion period. Analysis of metabolic activity and membrane integrity showed that CsA was unable to inhibit cell death (Figure 30a & b).

5μM BA was applied in the same manner as CsA, either as a pre-treatment, during ischaemic solution application, or during simulated reperfusion. After the 18hr of simulated reperfusion, the cells were analysed for metabolic activity and membrane integrity. As with t-BOOH, the effect of BA was extremely erratic, on some occasions it's application was extremely toxic to the cells. However, only on rare occasions could any inhibitory effect be seen (Figure 31a & b).

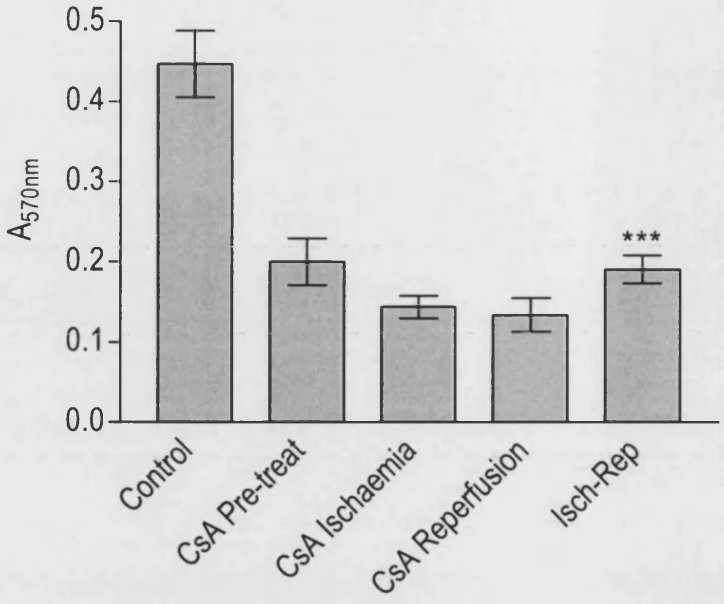


Figure 30a: 50nM cyclosporin A (CsA) was applied to primary cardiomyocytes, either for 1hr prior to the experiment, during application of ischaemic solution, or during the 18hr simulated reperfusion period. Analysis of metabolic activity showed no inhibitory effect of CsA [***, $p < 0.001$ v. control].

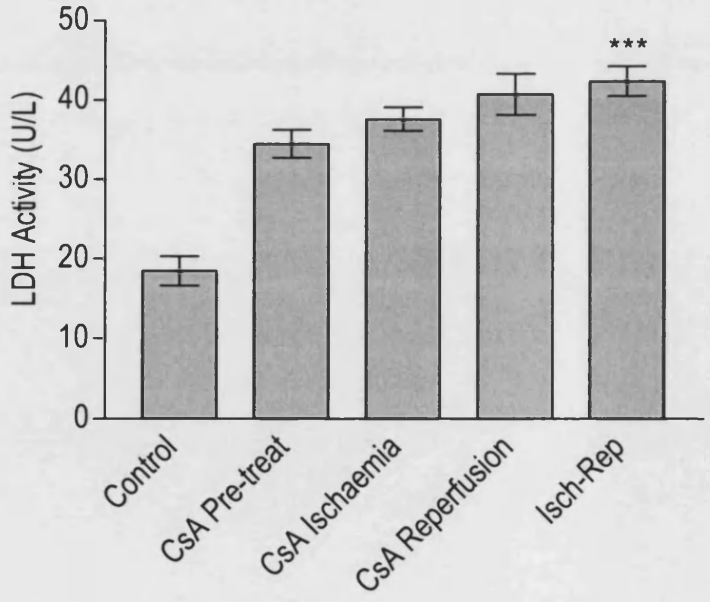


Figure 30b: The same cardiomyocytes assayed for LDH release showed that cyclosporin A

(CsA) was unable to inhibit the loss of membrane integrity induced by simulated ischaemia and reperfusion [***, $p < 0.001$ v. control].

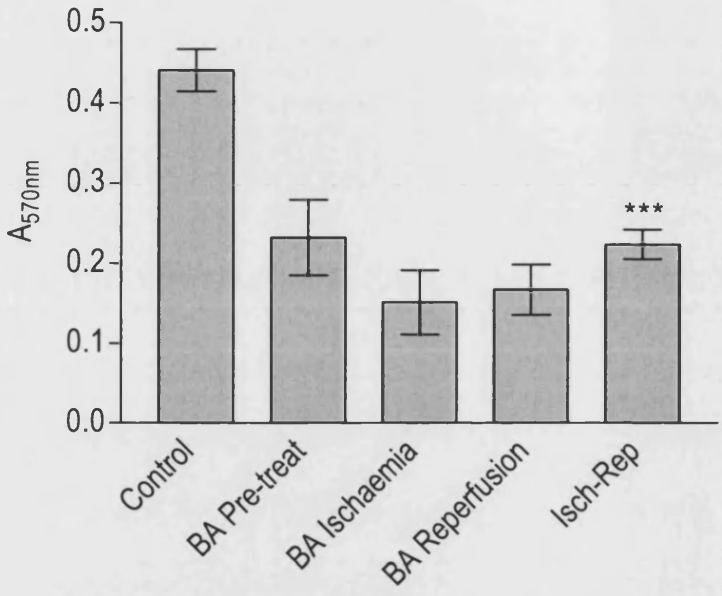


Figure 31a: 5μM bongkrekic acid (BA) was applied to cardiomyocytes, either for 1hr prior to the experiment, during treatment with the simulated ischaemia solution, or during the 18hr simulated reperfusion period. Analysis of metabolic activity quantified by conversion of MTT reagent showed no overall inhibitory effect of BA [***, $p < 0.001$ v. control].

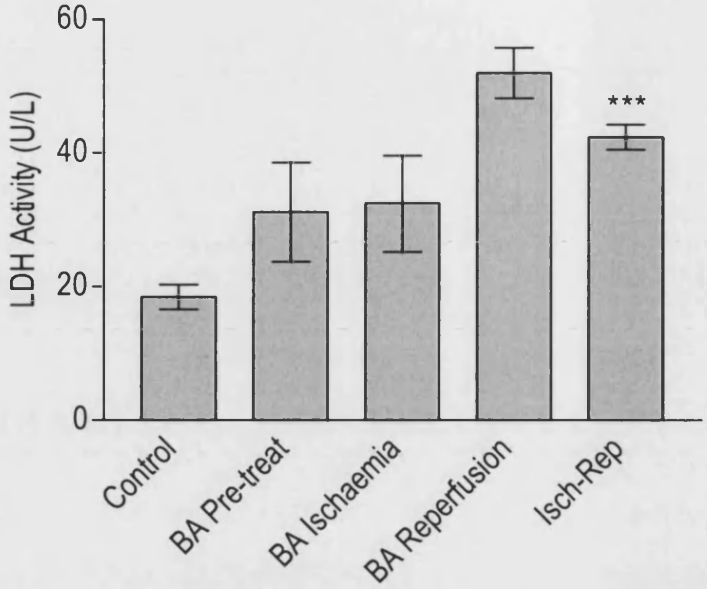


Figure 31b: The same cardiomyocytes assayed for LDH release showed that bongkrekic acid (BA) was unable to inhibit the loss of membrane integrity caused by 3hr simulated ischaemia solution treatment and 20hr simulated reperfusion [***, $p < 0.001$ v. control].

3.5 Discussion

As stated in Chapter 1 the problem of quantifying apoptosis is a contentious issue, with many of the available methods of detection such as TUNEL staining being heavily disputed. Additionally, many different pathways of apoptosis have been reported. The classical apoptotic features are ubiquitous in physiological apoptosis, but it is difficult to think of any event that has been reported in all cases of pathological apoptosis. Therefore, it cannot be maintained with certainty that apoptosis is being accurately measured and any attempt to differentiate accurately between apoptosis and necrosis must be viewed with some scepticism.

In this study, SSP was used to induce apoptosis. SSP is widely recognised as an effective inducer of apoptosis and has been utilised by many researchers. It has previously been used in a wide range of cell types including primary cardiomyocytes and the resulting apoptotic cell death has been measured by a wide variety of methods (12, 13, 39, 59, 233). As such it can be employed for the induction of apoptosis with relative confidence. In this study analysis by electron microscopy served to confirm that apoptosis was occurring in the cardiomyocyte cultures and also illustrated that high concentrations of SSP induced necrotic cell death instead of apoptosis.

For the quantification of apoptosis, three separate parameters were measured. Reduction of metabolic activity and plasma membrane integrity are features of all forms of cell death. In the case of apoptosis, metabolic activity must remain high in order to fully implement the process and loss of membrane integrity occurs only after the process is essentially complete. Both parameters must therefore be viewed as extreme endpoints. Fragmentation of nuclear DNA is commonly associated with apoptosis although it has also been reported in essentially necrotic cells (55). It occurs during the active process, prior to the formation of apoptotic bodies and should therefore be detectable before significant changes in metabolic activity and membrane integrity can be seen.

This is borne out by the results obtained. No significant change in metabolic activity or membrane integrity could be observed until after 18-24hr of SSP treatment. However, analysis of DNA fragmentation through the quantification of DNA loss from permeablised cells showed significant changes from as early as 4hr. Taken together these data show that primary cardiomyocytes begin undergoing apoptosis within 4 hours of application of SSP. As apoptosis is an active process metabolic activity remains high and as the plasma membrane is not compromised the release of LDH is low. However, at a later stage in the process when over 50% of the cells are dead and undergoing post-apoptotic necrotic changes, a corresponding decrease in metabolic activity and release of intracellular enzymes can be observed.

These data, combined with the known properties of SSP make it certain that apoptosis was the predominant form of cell death and subsequently, that the effects of the chosen interventions could be accurately measured. Finally, Western blotting of SSP-treated cells with an antibody against caspase 3 revealed cleavage of the 32kDa caspase 3 precursor protein that was not observed in the control cells. This finding is not in any way conclusive, since activation of caspases has been demonstrated in necrotic cell death as previously mentioned, and there could be other reasons for the disappearance of the caspase 3 precursor, such as a general breakdown of proteins in the dying cell. Although detection of the activated caspase 3 protein is a more direct means of showing caspase involvement, the available antibodies were not found to be reliable. However cleavage of the caspase 3 precursor protein serves as an extra piece of evidence for the activation of apoptotic pathways.

CsA, which binds to and inhibits cyclophilins, was applied to the cardiomyocytes as an aid to study the role of CyPD in cardiomyocyte cell death. The primary cardiomyocytes showed sensitivity to the toxic effects between 400 and 600nM CsA. This is in contrast with other cell types that are apparently resistant to much higher concentrations of CsA and reflects differences in cellular metabolism. For example primary rat hepatocytes do not show signs of

compromised membrane integrity when treated with up to 10μM CsA (214) and 30μM CsA was used without ill effect in human osteosarcoma cells (150). Long term CsA treatment is known to cause widespread tissue damage *in vivo* and causes apoptosis in cultured cells. The mechanisms underlying this toxicity appear to vary according to cell type. A number of studies have shown CsA induction of nitric oxide (213, 234, 235) and Esposito and colleagues reported CsA induced changes in cell proliferation, nitric oxide production and extra cellular matrix turnover, with the precise response to CsA being dependent upon the cell type (213).

When applied to primary cardiomyocytes prior to SSP treatment, CsA was toxic at a lower concentration than was observed without SSP. This is presumably due to interaction between CsA and SSP and is supported by the fact that when applied concurrently, CsA and SSP were toxic to the cells even at a CsA concentration as low as 50nM. When applied as a pre-treatment, 50nM CsA had no detectable effect on the extent of apoptosis. However, in the light of the results above, it is possible that there was a slight toxic effect that was undetectable by the methodology chosen. An alternative possibility is that 50nM CsA was too low to be an effective dose. In this instance it may not be possible to apply an effective concentration that will not be toxic in combination with SSP. It must also be considered whether the CsA was successfully entering the mitochondria and binding to CyPD and if it did, then whether this was effectively inhibiting the collapse of the mitochondrial transmembrane potential. An additional possibility is that collapse of the transmembrane potential is a consequence of SSP-induced apoptosis as opposed to a cause. In which case, inhibiting the MPTP would not affect the extent of apoptosis.

In order to clarify these results, BA was selected as an alternative inhibitor to CsA. BA did not display any unexpected toxicity either when applied as a pre-treatment, or concurrently with SSP. However, it displayed no discernable influence on the extent of apoptosis by any of the

measured parameters. BA directly inhibits the adenine nucleotide translocase (ANT) by binding to it and stabilising it in its closed 'M' conformation (236). Unlike CyPD, which is often described as a regulator of the MPTP, the ANT is believed to form at least part of the fundamental structure of the pore. Direct inhibition of the ANT, should therefore unquestionably inhibit formation of the MPTP and, theoretically, prevent the collapse of the transmembrane potential. However, stabilising the ANT in its closed conformation will also inevitably inhibit the passage of adenosine nucleotides across the mitochondrial membrane, thereby affecting cell metabolism. When applied for 2 hrs at a concentration of up to 10μM, BA did not have any significant effect on metabolic activity as measured by the conversion of MTT reagent. Neither, however, did BA have any significant effect on the progression of apoptosis. This could mean that BA did not successfully enter the cells, and was therefore unable to influence either metabolism or the MPTP. Alternatively, it may be that the low concentrations of BA used, that did not noticeably inhibit ATP movement, were also unable to influence formation of the MPTP, that is, after all, a pathological event. A further possibility is that the MPTP did not play a significant role in this model of apoptosis, so that cell death could not be influenced by the presence of BA.

From the data obtained in this chapter, it is not possible to determine the precise reason for the failure of CsA to inhibit apoptosis. A further explanation is that inhibition of the pore by CsA and BA is a short-lived event. It is possible that over the course of the 18hr SSP treatment the inhibition is overcome and the cells undergo apoptosis. Further experiments are required to examine the process in further detail. Specifically, it must be determined whether CsA is successfully binding to CyPD and whether either inhibitor is capable of entering the cells and preventing the collapse of mitochondrial transmembrane potential.

In order to establish a model of necrotic cell death, t-BOOH was applied to primary cardiomyocytes for 1hr. A short treatment period was chosen in part to address the question of whether MPTP inhibition by CsA and BA was only a transient event. Over the course of 1hr 100μM consistently produced significant but not total cell death and analysis by EM showed that the cells were dying by necrosis.

When applied either prior to or concurrently with t-BOOH, CsA did not display any unexpected toxicity. Effective inhibition of cell death could only be seen when CsA was applied concurrently with t-BOOH and at the low concentration of 50nM. This is contradictory to other studies that have stated 200nM or greater to be the most effective CsA concentration in rat primary cardiomyocytes. However the cardiomyocytes used in these studies were from older animals, the youngest used were from 2-week-old pups. Other studies have shown that the effective dose of CsA in primary cardiomyocytes varies according to the age of the animals used (133, 136, 237). Therefore it is not surprising that the results obtained in this study differed from existing reports. Doyle and colleagues showed that 500nM was the most effective dose of CsA in cells taken from 2-week-old pups, a concentration that would probably have been toxic in cells from 1-3 day olds (significant toxicity was noted between 400 & 600nM). Additionally the group applied 500μM t-BOOH to their cells for over 70min (237), a treatment that killed the entire population of our cells in less than 1hr. It is therefore probable that cardiomyocytes from 1-3 day old rats were more sensitive to the application of chemicals than those used in other studies. Thus a much lower effective dose of CsA was observed.

BA proved to be extremely toxic when applied in combination with t-BOOH. When applied as a pre-treatment it was not significantly toxic but no inhibition of cell death could be seen. It is probable that this toxicity was due to interaction between BA and t-BOOH and even the most rigorous washing prior to t-BOOH application could not completely prevent the reaction.

Many previous studies have utilised solutions designed to mimic the *in* vivo conditions of ischaemia *in vitro* (238-242). Such a system has several advantages. A far greater range of parameters can be easily studied in a cell culture system than *in vivo* and a single cell type can be easily studied. A greater range of interventions can be applied without the ethical considerations encountered in animal work and *in vitro* work is invariably less expensive. However, there are also problems associated with *in vitro* simulations. There may be varying opinions of the precise conditions encountered during *in vivo* ischaemia leading to argument over the content of the simulation buffers used. Additionally there may be other factors involved *in vivo* which are as yet unknown and therefore cannot be accounted for in a simulation. Such simulations therefore are useful tools for studying the mechanisms behind ischaemia-reperfusion injury, allowing more detailed analysis than can be easily achieved in whole tissue. However they can only serve as a starting point and the results cannot necessarily be extrapolated to an *in vivo* situation.

The solution used in this instance is based upon a protocol of Esumi and colleagues (217) incorporating slight modifications by Dr. R. Heads (personal communication). The solution is based on Krebs buffer but includes 2-deoxyglucose to inhibit glycolysis and sodium dithionite to inhibit mitochondrial respiration and scavenge oxygen. The addition of lactate and KCl mimics the conditions present during ischaemia *in vivo* (see introduction). The oxygen scavenging effect of sodium dithionite decreases after approximately 1hr so the cells were placed in a hypoxic chamber to maintain the anoxic environment for the duration of the experiment.

This treatment caused a time-dependent decrease in cell viability as expected. Also as expected, subsequent incubation in full media caused a further decrease in viability, a gradual decline in metabolic activity over the first 18 hours followed by a sharp decrease between 18

and 24 hours of simulated reperfusion. This coincided with a significant increase of LDH activity in the cell media showing that plasma membrane integrity was severely compromised. When the cells were examined by electron microscopy, it could be clearly seen that they were dying by necrosis and were in fact in a state of greater degradation than those treated by t-BOOH. This was certainly due to the longer treatment period.

These results would initially suggest that the system used was successfully simulating ischaemia-reperfusion injury at least to some degree.

Treatment with CsA and BA did not produce the expected results. It has been shown on many occasions that the MPTP opens during reperfusion. Therefore inhibitors of the MPTP would be expected to reduce the extent of cell death during the simulated reperfusion phase if not during application of the ischaemic solution. CsA and BA were applied during all phases of the experiment but no influence was observed. On some occasions BA was in fact toxic if applied as a pre-treatment or concurrently with the simulated ischaemia solution. Presumably BA was reacting with an element of the ischaemic solution, although if that is the case it is difficult to explain why it was not always toxic when applied together with the ischaemic solution. CsA did not display any obvious toxicity but nevertheless failed to inhibit the loss of cell viability. As CsA had a significant effect on t-BOOH induced necrosis, it is likely that CsA is successfully binding to CyPD and inhibiting induction of the MPTP. However it still failed to reduce cell death during application of the simulated ischaemia solution. One possibility, as with the SSP model is that the experimental time is too long and the effect of CsA is only transient. Alternatively, it may be that the model of simulated ischaemia is not an accurate representation of the *in vivo* situation and the importance of the MPTP is diminished. It is therefore important to directly assess the effect of CsA on the behaviour of the MPTP in this model.

3.6 Conclusions

Models of apoptosis, necrosis and simulated ischaemia-reperfusion injury were successfully implemented in neonatal primary cardiomyocytes.

Application of MPTP inhibitors only slightly suppressed necrotic cell death and was ineffective in the cases of apoptosis and simulated ischaemia-reperfusion injury. Therefore further study is required to clarify the situation.

4: Quantification of mΔΨ and the Effect of MPTP Inhibition

4.1 Introduction

Translocation of protons across the mitochondrial membranes down the electron transport chain creates an electrochemical gradient with a potential gradient of approximately –180mV. This mitochondrial transmembrane potential, generally referred to as the mΔΨ, is the driving force for ATP synthesis and therefore crucial to the health of the whole cell. Opening of the mitochondrial permeability transition pore (MPTP) for any prolonged period inevitably disrupts this gradient resulting in a decline in ATP synthesis. For this reason, induction of the MPTP is widely recognised as an important factor in many situations of cell injury and death.

In particular, its role in ischaemia-reperfusion injury has attracted attention as it has been shown that opening of the MPTP upon reperfusion may be in part responsible for the increase in cell injury. A large number of studies have pinpointed MPTP induction as a crucial point in cell death (7, 118, 243) but equally there have been studies reported that MPTP was not induced or its influence was unimportant (150, 187). Although the methods for detecting MPTP induction are often disputed, there can now be little doubt that it's function and relative importance varies widely according to the cell type and manner of death induction.

4.2 Objective

In a study dealing with the influence of the MPTP of cell death it is crucial to measure induction of the pore directly, and if possible unambiguously. The aim of this study was to measure mΔΨ in primary cardiomyocytes using the 3 models of cell death described in the previous chapter.

Additionally the effect of inhibitors of the MPTP on mΔΨ was examined.

4.3 Materials and Methods

Neonatal primary cardiomyocytes were isolated and cultured as described in chapter 2.2.1. Cell death was induced using staurosporine (SSP), t-butylhydroperoxide (t-BOOH) and a solution designed to replicate the extracellular conditions of ischaemia-reperfusion injury as detailed in chapter 2.2.2-4.

Mitochondrial transmembrane potential ($m\Delta\Psi$) was measured using the fluorescent dye 3,3'dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and analysed by flow cytometry as described in chapter 2.4.1 or by confocal microscopy as detailed in 2.4.2.

4.4 Results

4.4.1 Measurement of mΔΨ in primary cardiomyocytes

Fluorescence of ($DiOC_6(3)$) was measured by flow cytometry and plotted against cell size as measured by forward scatter. Brightly stained cells again correlated with normal size and granularity and although the pattern of staining with ($DiOC_6(3)$) was more diffuse than the propidium iodide (PI) staining described in chapter 3.4.2, 1 group of small, very poorly stained cells were seen as well as 2 groups of larger, brightly stained cells which were assumed to be the viable cell population (see Figure 32). This allowed quantification of the percentage of cells with viable mitochondria as well as separate assessment of the mΔΨ of the viable cell population.

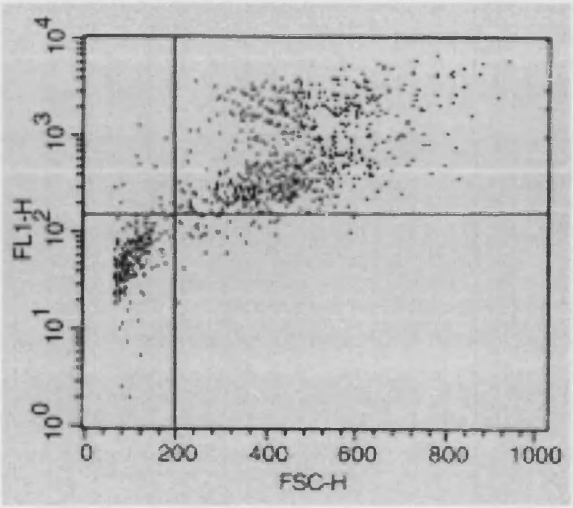


Figure 32: Control cardiomyocytes stained with ($DiOC_6(3)$) were analysed on a flow cytometer and cell size (FSC-H) was plotted against fluorescence (FL1-H) in a scatter plot, an example of which is shown above. This revealed one group of small, unstained cells and 2 groups of large, brightly stained viable cells. The quadrant parameters were set to contain all of the apparently viable cells in the upper-right quadrant. These cells were then analysed either by number or by mean fluorescence intensity.

In order to demonstrate that ($DiOC_6(3)$) was genuinely measuring mΔΨ, primary cardiomyocytes were treated for 1hr with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Fluorescence of ($DiOC_6(3)$) was then measured as above and expressed as a percentage of the control. Analysis of the number of viable cells showed no variation between the control and treated cells, due to the fact that the cell size was unaffected by CCCP treatment (Figure 33a). Measurement of the geometric mean fluorescence of the total cell population (including a negative staining control) also failed to show any change in staining (Figure 33b). This was explained by the fact that the two groups of cells seen in Figure 32 created two main peaks of fluorescence when plotted in a histogram (Figure 33c). During treatment the cells moved from the brightly stained group into the poorly stained group without creating any significant changes in the geometric mean, which lies midway between the two groups. However, when the mean fluorescence intensity (MFI) of the total cell population was measured, a significant and concentration-dependent decrease was observed (Figure 33d) showing that fluorescence of ($DiOC_6(3)$) was relative to mΔΨ. Quadrant markers were set on the control samples to exclude the unstained cells (as shown in figure 32) allowing independent measurement of the viable population. This proved to be a far more sensitive method of analysis. A highly significant concentration dependent reduction in both the geometric mean (Figure 33e) and the MFI (Figure 33f) was observed.

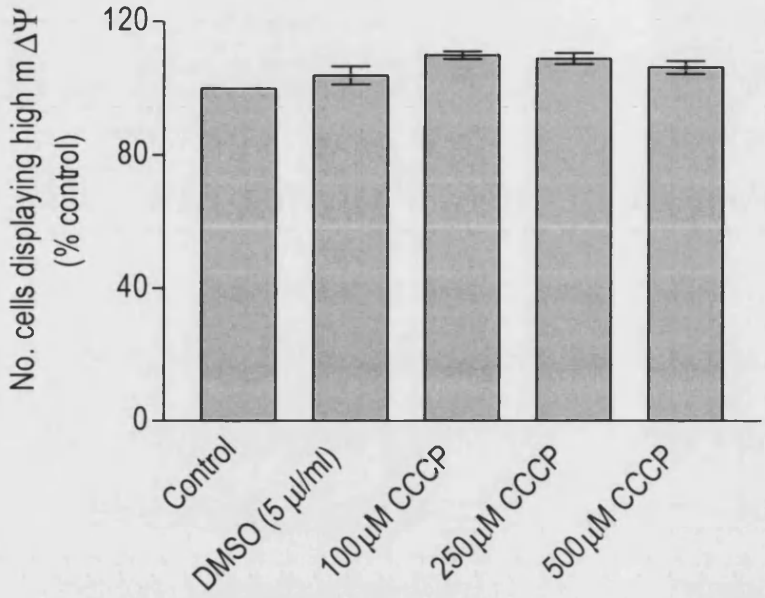


Figure 33a: Cardiomyocytes treated with the mitochondrial uncoupler CCCP were stained with ($DiOC_6(3)$) and the number of cells in the upper-right quadrant was counted (see Figure 32). This revealed no variation between control and treated cells.

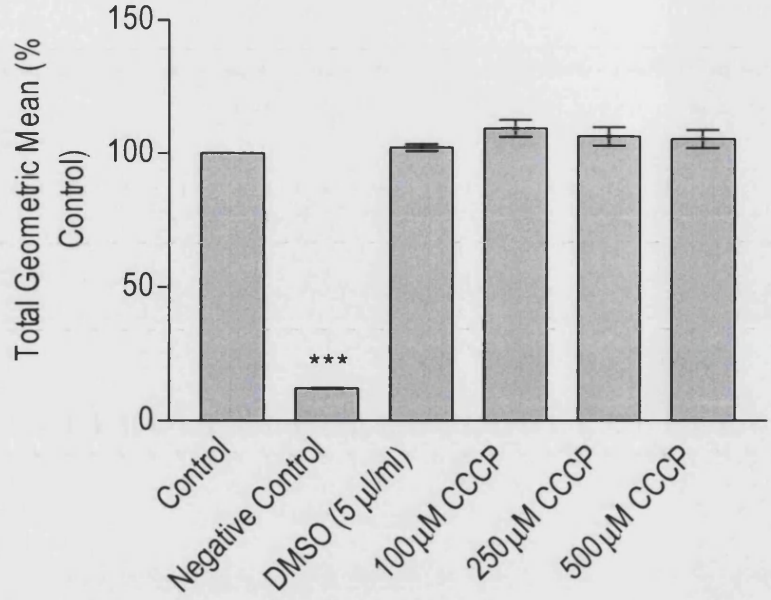


Figure 33b: Analysis of the geometric mean of the total cell population in the same samples also failed to show any significant changes in ($DiOC_6(3)$) fluorescence after treatment with the mitochondrial uncoupler CCCP [***, $p < 0.001$ v. Control].

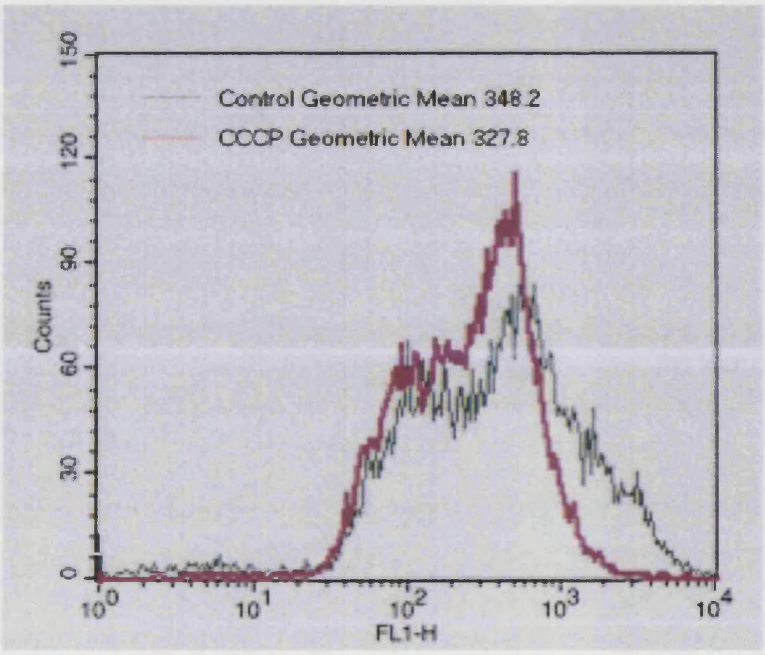


Figure 33c: This representative histogram shows the ($DiOC_6(3)$) fluorescence of control and CCCP-treated cells. This demonstrates that the geometric mean remains changes only slightly despite clear changes in the distribution of fluorescence.

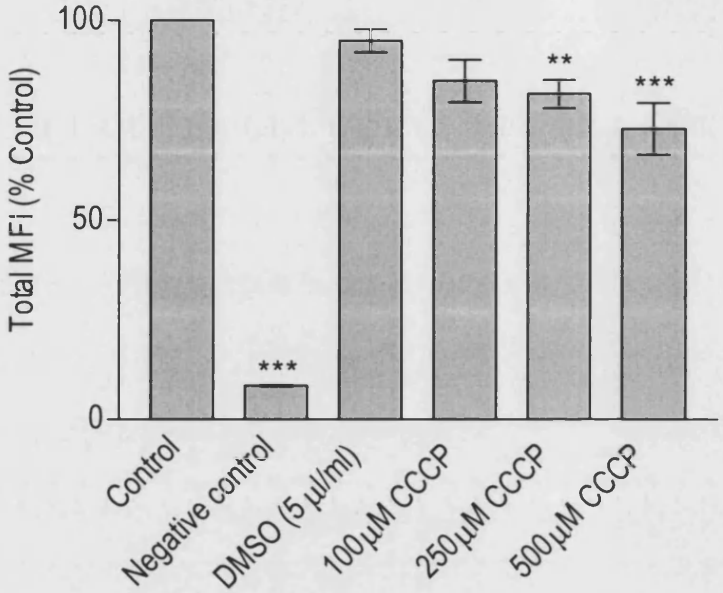


Figure 33d: Analysis of the mean fluorescence intensity (MFI) of the total cell population of the same cells treated with the mitochondrial uncoupler CCCP revealed a significant concentration-dependent decrease in ($DiOC_6(3)$) fluorescence [**, $p < 0.01$ v. Control; ***, $p < 0.001$ v. Control].

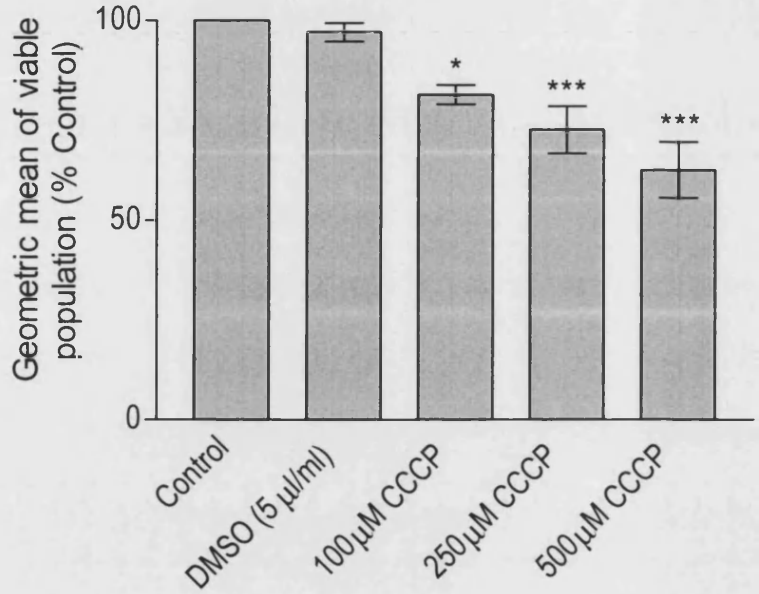


Figure 33e: Analysis of the geometric mean of only the viable cell population after application of the mitochondrial uncoupler CCCP revealed a concentration-dependent decrease in ($DiOC_6(3)$) fluorescence of greater significance than the in the total population [*, $p < 0.05$ v. Control; ***, $p < 0.001$ v. Control].

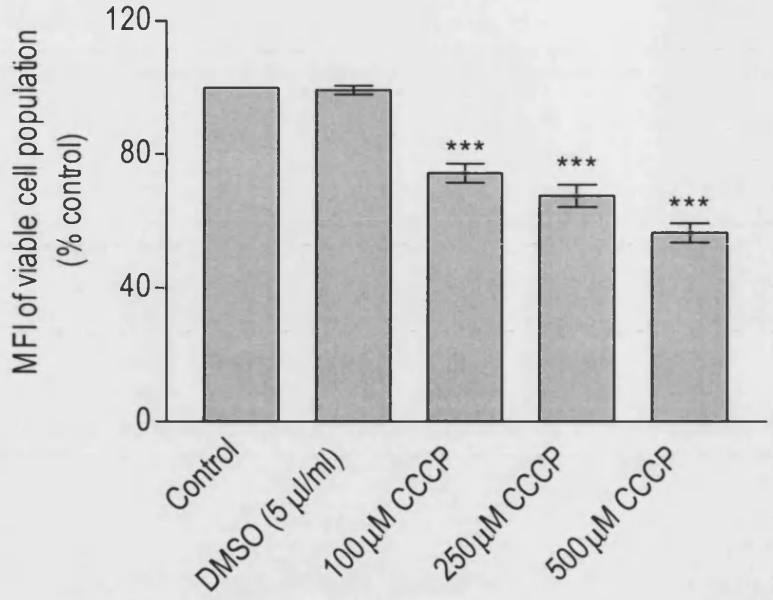


Figure 33f: Analysis of the MFI of the viable cell population revealed a ($DiOC_6(3)$) concentration-dependent decrease in fluorescence following application of the mitochondrial uncoupler CCCP that was more highly significant than the geometric mean [***, $p < 0.001$ v. Control].

Primary cardiomyocytes stained with ($DiOC_6(3)$) were visualised by confocal microscopy. When 20nM ($DiOC_6(3)$) was used, staining of the nuclear membrane and some random diffuse fluorescence was seen in addition to discrete staining of small sub-cellular compartments (Figure 34a). However, when a concentration of less than 1nM was used (0.8nM) as recommended by Rottenberg and colleagues (222), no nuclear membrane staining was seen (Figure 34b). Upon application of 1µM of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), the discrete staining was abolished and the fluorescence became diffused throughout the cell as illustrated in Figures 35 & 36. This demonstrated that ($DiOC_6(3)$) was specifically staining the mitochondria.

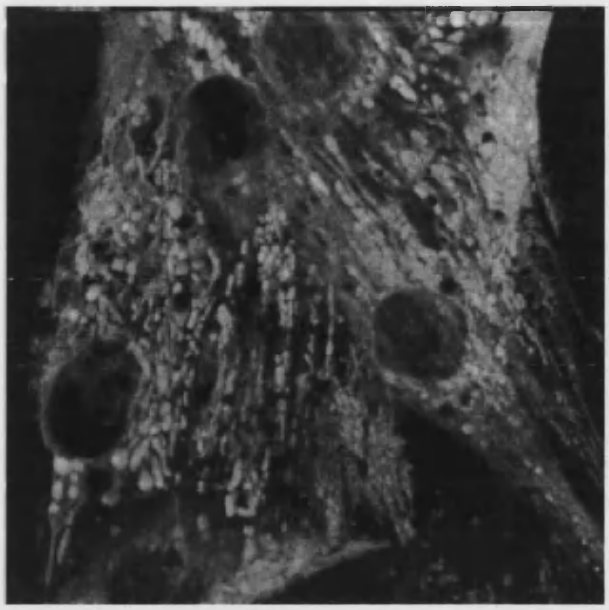


Figure 34a: Primary cardiomyocytes were stained with 20nM ($DiOC_6(3)$) and visualised by confocal microscopy. Discrete staining of small sub-cellular compartments can be seen but additionally, the nuclear membrane is defined and some diffuse fluorescence can be observed in the cytoplasm.

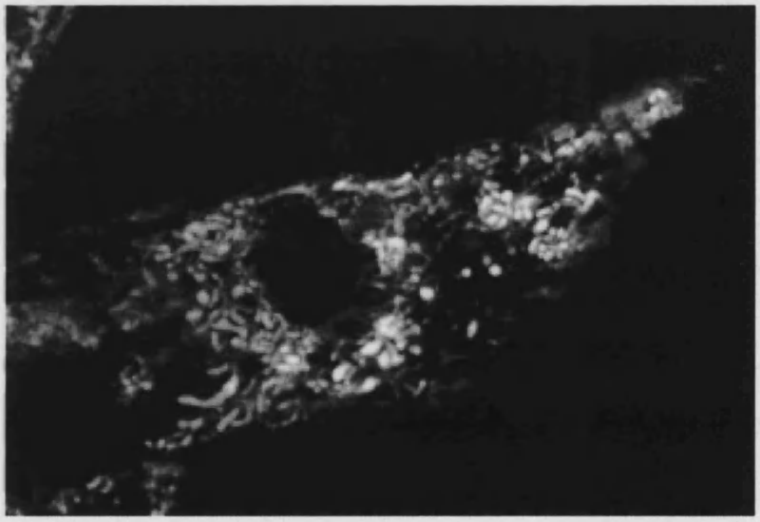


Figure 34b: Primary cardiomyocytes were stained with 0.8nM ($DiOC_6(3)$) and visualised by confocal microscopy. Discrete staining of small sub-cellular compartments can be seen with no definition of the nuclear membrane or diffuse cytoplasmic fluorescence.

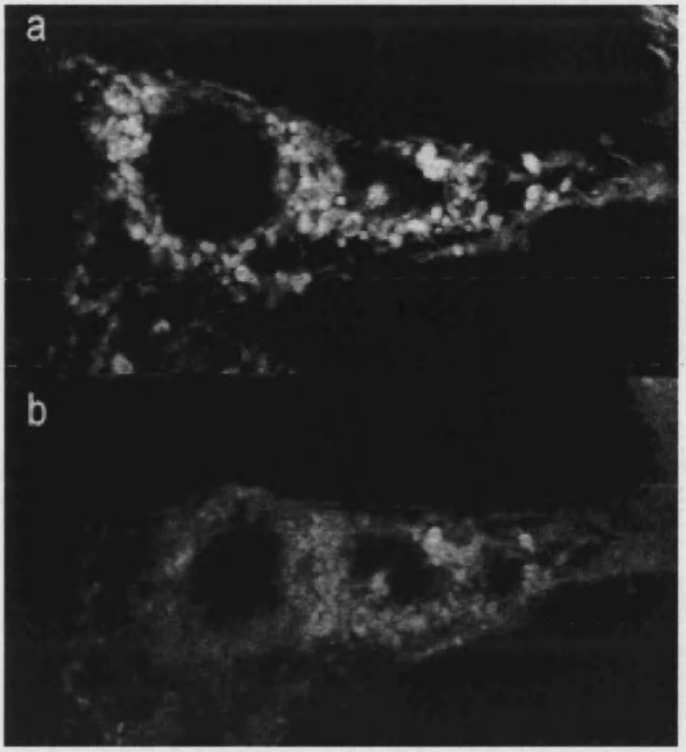


Figure 35: (a) Primary cardiomyocytes were stained with 0.8nM ($DiOC_6(3)$) and visualised by confocal microscopy, (b) in cardiomyocytes photographed 30 seconds after the addition of 1μM of the mitochondrial uncoupler FCCP the discrete staining was lost and the fluorescence had diffused throughout the cell.

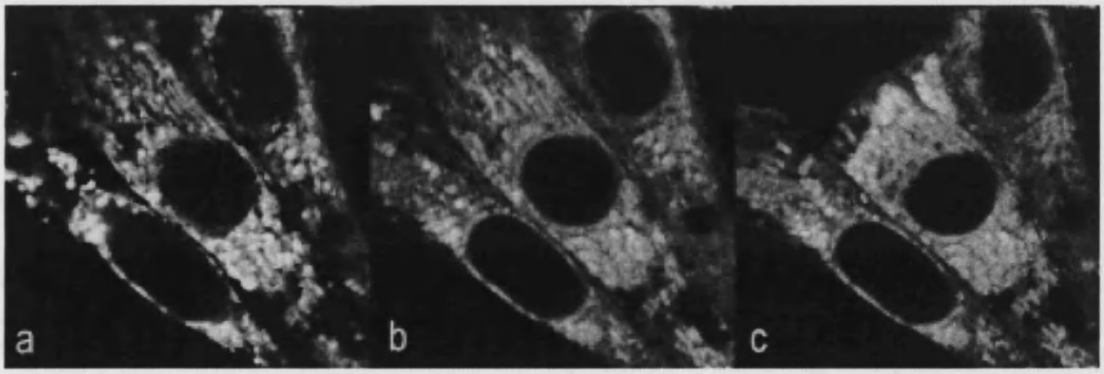


Figure 36: In a certain experiment (a, photographed prior to FCCP treatment) the three cardiomyocytes lost the discrete staining (b, photographed 30 seconds after addition of FCCP) and the middle cell of the three was then seen to undergo rigor (c, photographed after a further 30 seconds)

4.4.2 Measurement of mΔΨ during apoptosis

The data presented above showed that ($DiOC_6(3)$) was successfully detecting changes in mΔΨ. Therefore, mΔΨ was measured in primary cardiomyocytes (see Figure 32) subjected to treatment with the apoptosis inducer SSP and changes in the number of cells displaying a high mΔΨ (i.e. as control) were monitored.

Application of 5μM SSP to primary cardiomyocytes caused a time-dependent reduction in the number of cells displaying high mitochondrial transmembrane potential as shown in Figure 37a. However when the mean fluorescence of the viable population was measured, no decrease in staining was observed as shown in Figure 37b.

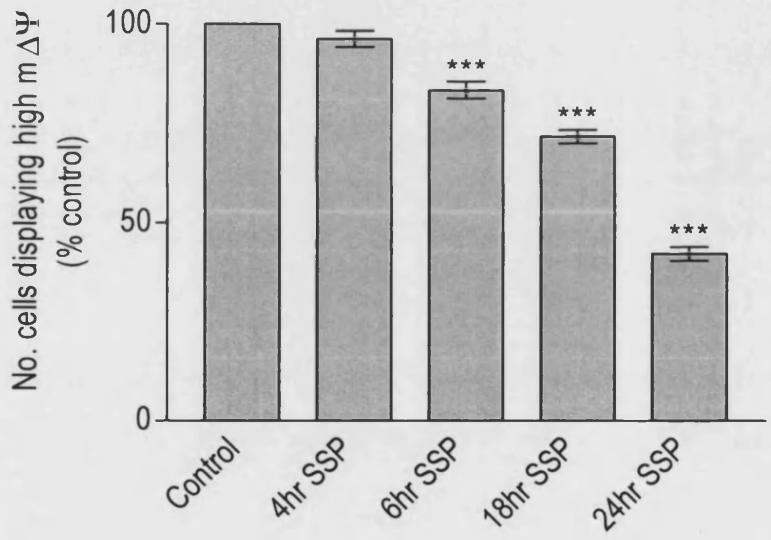


Figure 37a: Application of 5μM staurosporine (SSP) over 24hr caused a time-dependent reduction in the number of cells displaying high mitochondrial transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) [***, $p < 0.001$ v control].

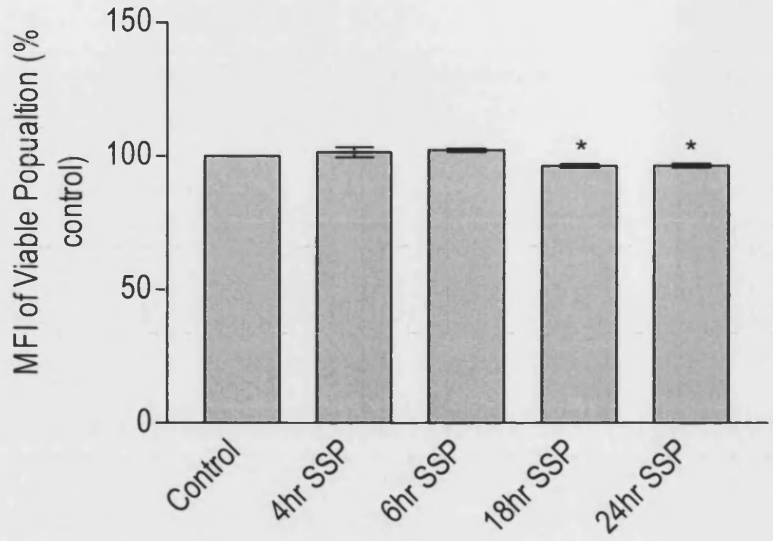


Figure 37b: The mean fluorescence intensity (MFI) of the viable cardiomyocyte population (upper-right quadrant, see Figure 32) remained comparatively high throughout the staurosporine (SSP) treatment period showing that mitochondria were taking up ($DiOC_6(3)$) and transmembrane potential was therefore little affected [*, $p < 0.05$ v control].

4.4.3 Measurement of mΔΨ during necrosis

Mitochondrial transmembrane potential was also assessed in primary cardiomyocytes following 1hr application of 100μM t-BOOH. This produced a significant drop in the number of cells displaying high fluorescence (Figure 38a) and furthermore, the uptake of ($DiOC_6(3)$) by the remaining viable cells was significantly reduced (Figure 38b).

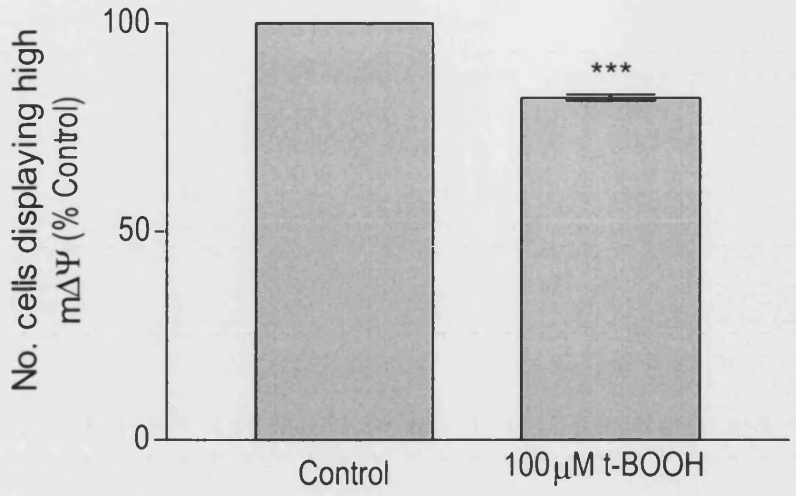


Figure 38a: Application of 100μM t-butylhydroperoxide (t-BOOH) for 1hr caused a significant reduction in the number of cells displaying high mitochondrial transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) [***, $p < 0.001$ v. control].

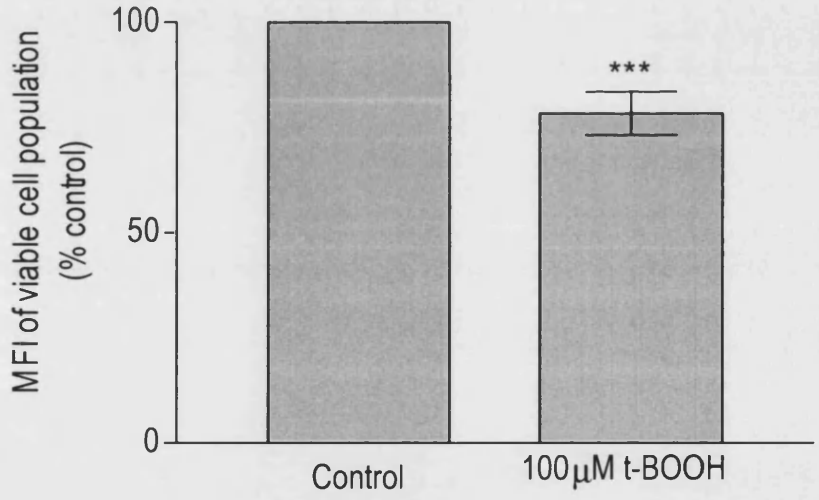


Figure 38b: During the 1hr of t-butylhydroperoxide (t-BOOH) treatment, the mean fluorescence intensity (MFI) of the viable cell population was significantly reduced showing that mitochondrial uptake of ($DiOC_6(3)$) was reduced and transmembrane potential has therefore declined [***, $p < 0.001$ v. control].

4.4.4 Measurement of mΔΨ during simulated ischaemia-reperfusion injury

Primary cardiomyocytes subjected to simulated ischaemia and reperfusion as described previously were analysed for changes in mΔΨ. After 3 hours of treatment with the simulated ischaemia solution there was no reduction in fluorescence, and for up to 4hr of simulated reperfusion no significant decrease in mΔΨ was observed; however after 20hr there was a significant decrease in fluorescence (Figure 39a & b).

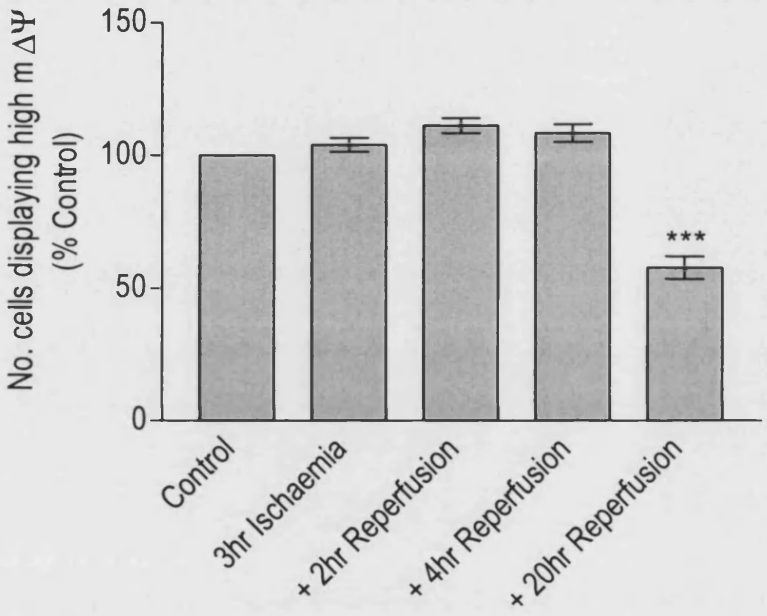


Figure 39a: 3hr application of the simulated ischaemia solution did not affect the number of cells displaying high mitochondrial transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) but after 20hr of simulated reperfusion the number was significantly reduced [***, $p < 0.001$ v. control].

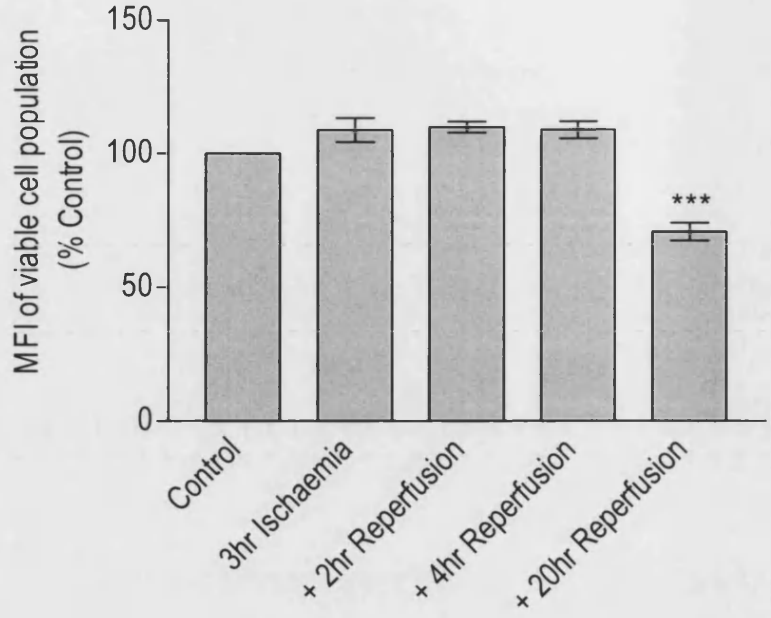


Figure 39b: After 3hr of simulated ischaemia, the mean fluorescence intensity (MFI) of the viable cell population was slightly higher than the control, but after 20hr of simulated reperfusion it was significantly reduced showing that mitochondrial uptake of ($DiOC_6(3)$) was reduced and transmembrane potential has therefore declined [***, $p < 0.001$ v. control].

4.4.5 The effect of MPTP inhibition on mΔΨ during apoptosis

Primary cardiomyocytes were treated with 50nM cyclosporin A (CsA) for 1hr prior to SSP application. Analysis of mΔΨ showed that CsA significantly inhibited the reduction of the number of cells with high membrane potential and also caused an increase in the membrane potential of the viable cell population (Figure 40a & b).

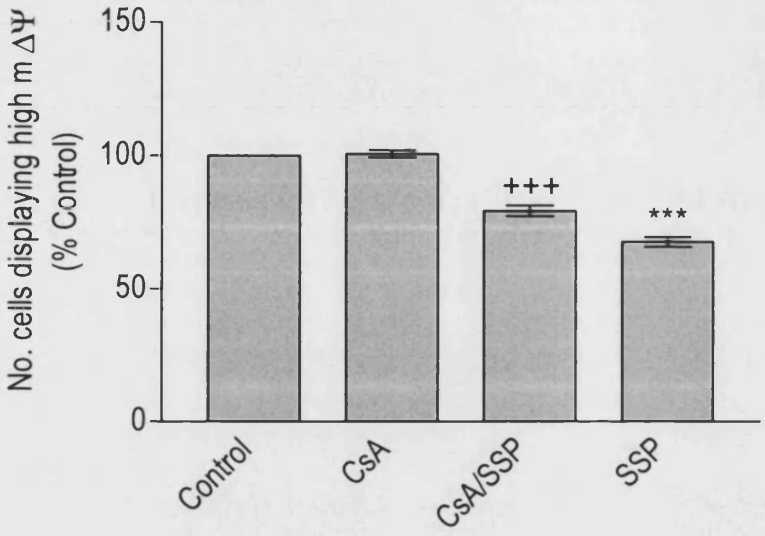


Figure 40a: 50nM cyclosporin A (CsA) application to cardiomyocytes prior to treatment with 5μM staurosporine (SSP) significantly inhibited the reduction of cells displaying high transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) [***, $p < 0.001$ v. control; +++, $p < 0.05$ v. SSP].

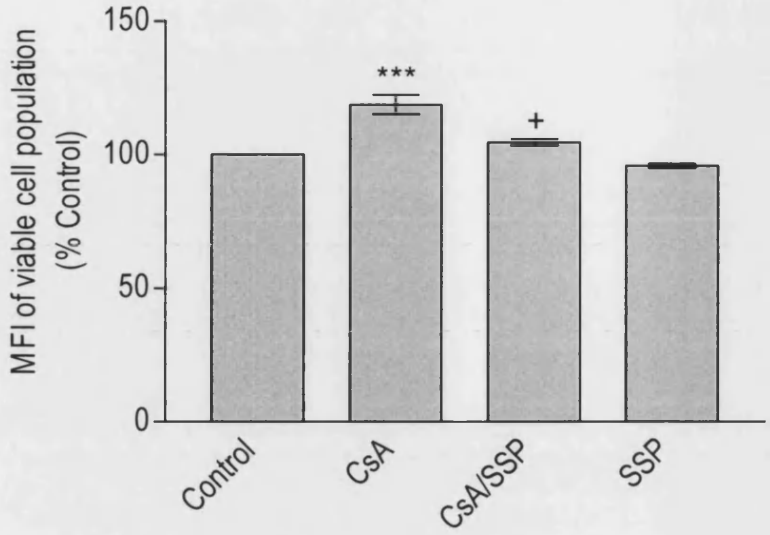


Figure 40b: Analysis of the viable population showed that cyclosporin A (CsA) pre-treatment caused a small but significant increase in the mean fluorescence intensity (MFI) of the viable cell population [***, $p < 0.001$ v. control; +, $p < 0.05$ v. SSP].

Primary cardiomyocytes were also treated with bongkrekic acid (BA) prior to SSP application. Analysis of mΔΨ revealed that BA significantly inhibited the reduction of the number of cells with high membrane potential and also caused an increase in the membrane potential of the viable cell population (Figure 41a & b).

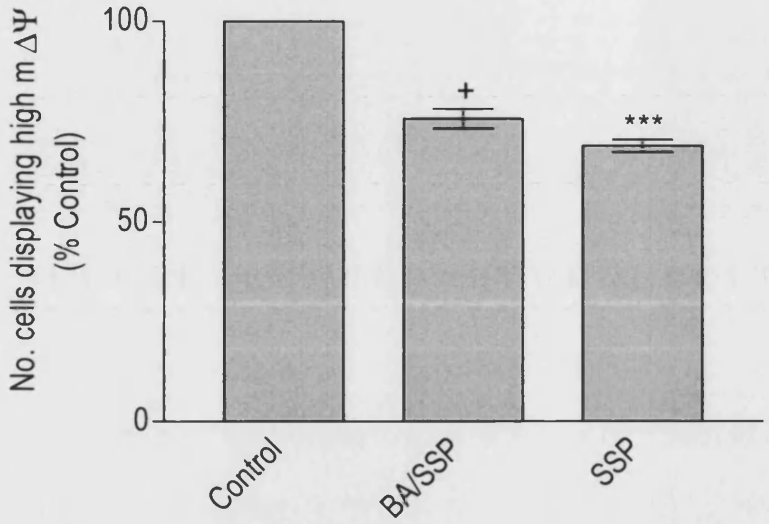


Figure 41a: Application of 5μM bongkrekic acid (BA) to cardiomyocytes prior to treatment with 5μM staurosporine (SSP) significantly inhibited the reduction of cells displaying high transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) [***, $p < 0.001$ v. control; +++, $p < 0.05$ v. SSP].

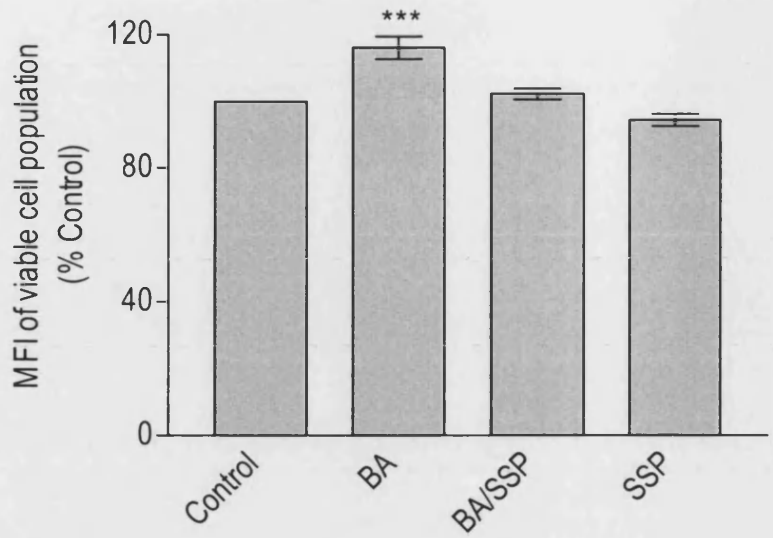


Figure 41b: Analysis of the viable population showed that 5μM bongkrekic acid (BA) pre-treatment also caused a small but significant increase in the mean fluorescence intensity (MFI) of the viable cell population [*, $p > 0.05$ v. control; +++, $p < 0.001$ v. SSP].

4.4.6 The effect of MPTP inhibition on mΔΨ during necrosis

50nM CsA was applied to primary cardiomyocytes prior to treatment with 100μM t-BOOH and analysis of mΔΨ. Quantification of the reduction of viable cells with high mΔΨ showed a slight but significant inhibition by CsA (Figure 42a), and analysis of the decrease in fluorescence in the viable population revealed highly significant inhibition by CsA (Figure 42b).

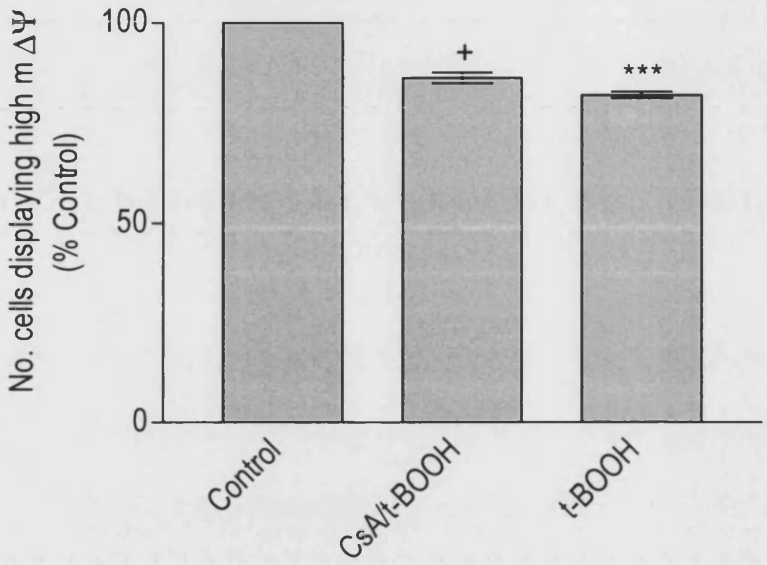


Figure 42a: Application of cyclosporin A (CsA) to cardiomyocytes prior to treatment with t-butylhydroperoxide (t-BOOH) significantly inhibited the reduction of cells displaying high transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) [***, $p < 0.001$ v. control; +, $p < 0.05$ v. t-BOOH].

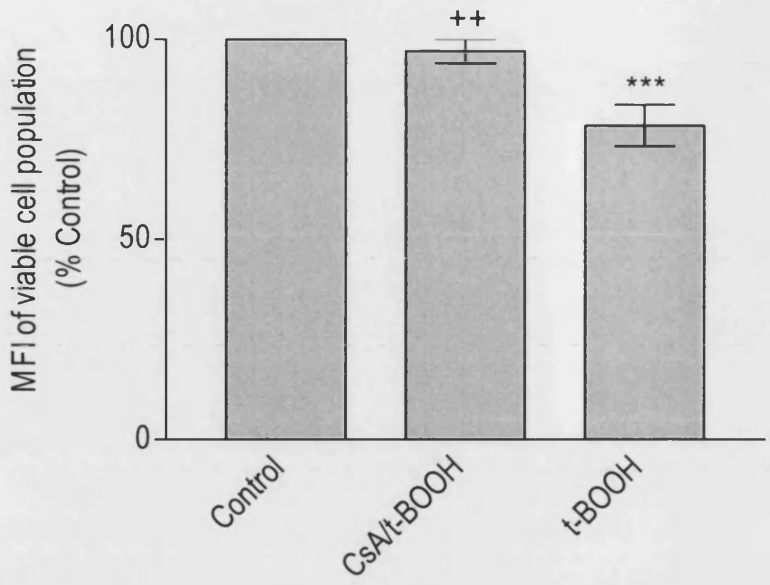


Figure 42b: Analysis of the viable population showed that cyclosporin A (CsA) significantly inhibited the reduction of the mean fluorescence intensity (MFI) caused by t-butylhydroperoxide (t-BOOH) showing that mitochondrial uptake of ($DiOC_6(3)$) was improved and the decline in transmembrane potential had therefore been inhibited [***, $p < 0.001$ v. control; ++, $p < 0.01$ v. t-BOOH].

4.4.7 The effect of MPTP inhibition on mΔΨ during simulated ischaemia-reperfusion injury

50nM CsA was applied to primary cardiomyocytes for 1hr prior to subjection to simulated ischaemia-reperfusion injury. CsA pre-treatment significantly inhibited both the reduction in the number of cells displaying high transmembrane potential and the decrease in the MFI of the viable population (Figure 43a & b).

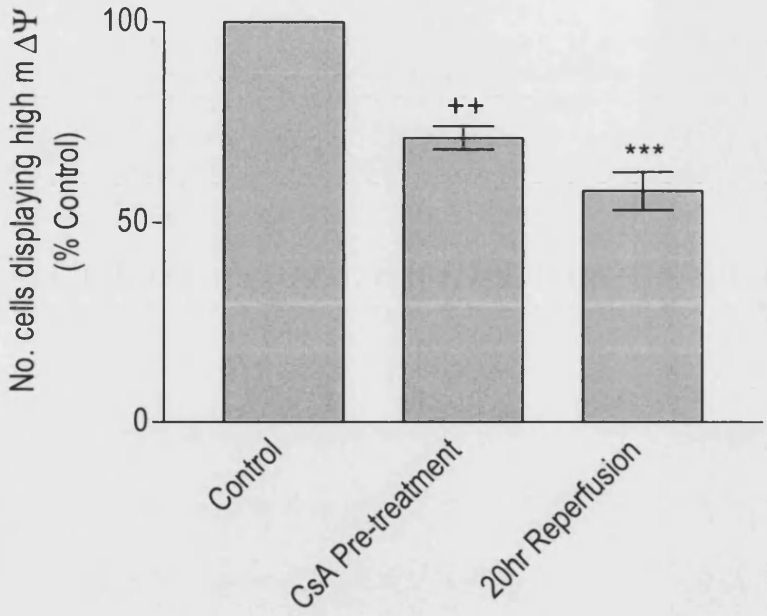


Figure 43a: Pre-treatment of cardiomyocytes with 50nM cyclosporin A (CsA) significantly inhibited the simulated ischaemia-reperfusion injury induced reduction in the number of cardiomyocytes displaying high transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) [***, $p < 0.001$ v. control; ++, $p < 0.01$ v. 20hr Reperfusion].

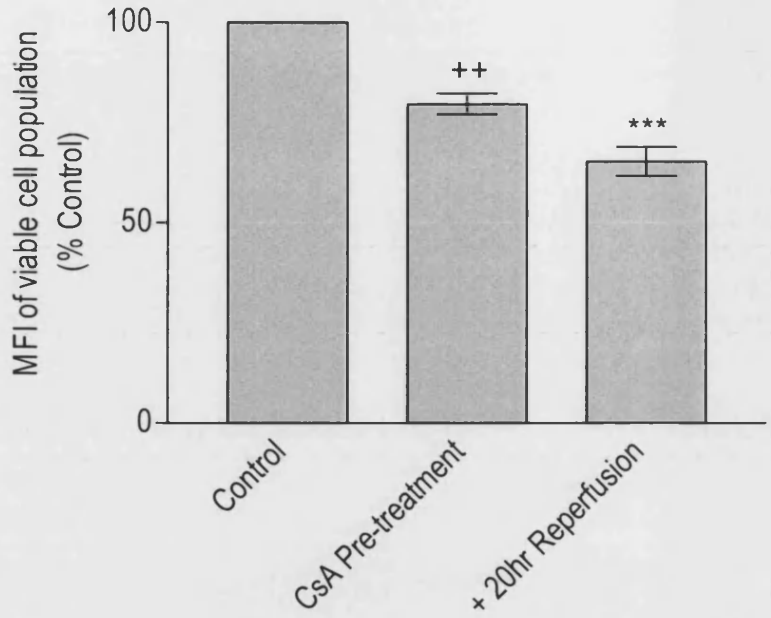


Figure 43b: Analysis of the viable population showed that 50nM cyclosporin A (CsA) significantly inhibited the reduction of the mean fluorescence intensity (MFI) caused by simulated ischaemia-reperfusion injury showing that mitochondrial uptake of ($DiOC_6(3)$) was improved and the decline in transmembrane potential had therefore been inhibited [***, $p < 0.001$ v. control; ++, $p < 0.01$ v. 20hr Reperfusion].

BA was applied to primary cardiomyocytes for 1hr prior to subjection to simulated ischaemia-reperfusion injury. BA pre-treatment significantly inhibited both the reduction in the number of cells displaying high transmembrane potential and the decrease in the MFI of the viable population (Figure 44a & b).

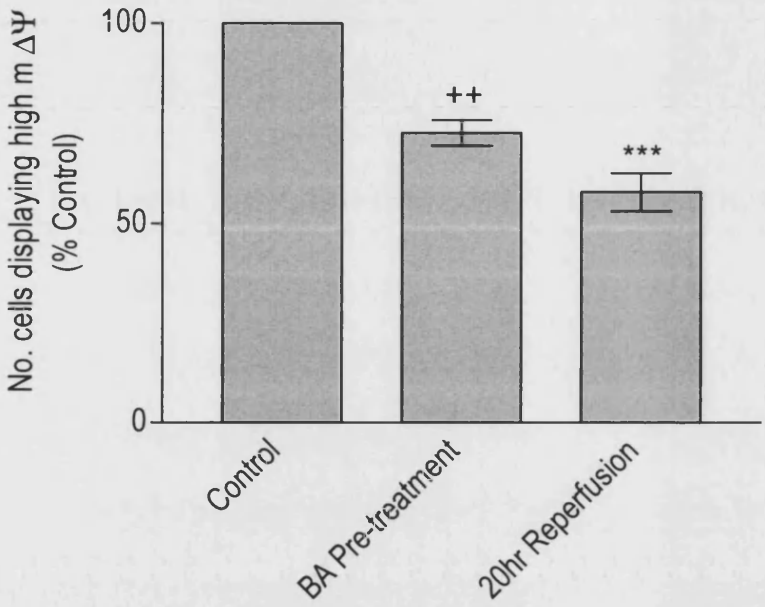


Figure 44a: Pre-treatment of cardiomyocytes with 5μM bongkrekic acid (BA) significantly inhibited the simulated ischaemia-reperfusion injury induced reduction in the number of cardiomyocytes displaying high transmembrane potential (mΔΨ) as quantified by uptake of ($DiOC_6(3)$) [***, $p < 0.001$ v. control; ++, $p < 0.01$ v. 20hr Reperfusion].

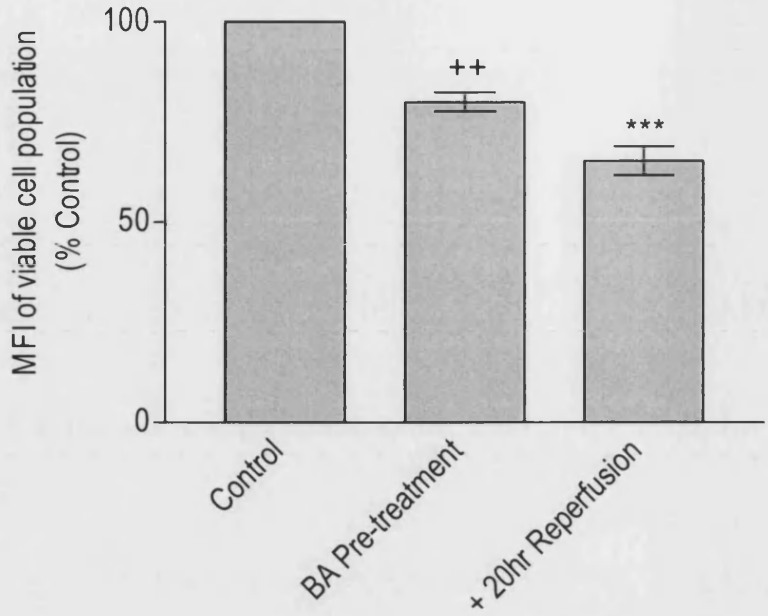


Figure 44b: Analysis of the viable population showed that 5μM bongkrekic acid (BA) significantly inhibited the reduction of the mean fluorescence intensity (MFI) caused by simulated ischaemia-reperfusion injury showing that mitochondrial uptake of ($DiOC_6(3)$) was improved and the decline in transmembrane potential had therefore been inhibited [***, $p < 0.001$ v. control; ++, $p < 0.01$ v. 20hr Reperfusion].

4.5 Discussion

Although mΔΨ-sensitive dyes have been used in many studies for the detection of MPTP induction, it is nevertheless a contentious area. Studies of these dyes have produced contradictory results so there is little consensus regarding their experimental use. In this study, ($DiOC_6(3)$) was chosen to evaluate mΔΨ in primary cardiomyocytes as this is one of the most widely utilised dyes and has previously been used in cardiomyocytes. Initial studies of this dye used a concentration of 40nM (221), a concentration which did not appear to be effective in cardiomyocytes (244). However, a more recent study by Rottenberg and Wu showed that an extremely low concentration of ($DiOC_6(3)$) (<1nM) was necessary for the accurate measurement of mΔΨ (222).

Evaluation of the ($DiOC_6(3)$)-staining of primary cardiomyocytes by confocal microscopy showed bright staining of discrete areas of the cells. At 20nM ($DiOC_6(3)$), nuclear membrane staining was seen which concurs with the findings of Rottenberg and colleagues. At 0.8nM ($DiOC_6(3)$) no nuclear membrane was observed and the discrete staining that was seen disappeared upon application of FCCP. This showed that ($DiOC_6(3)$) was being taken up into viable mitochondria and diffused out upon the collapse of mΔΨ. When this effect was quantified using CCCP and flow cytometric analysis, a concentration-dependent decrease in fluorescence was observed further supporting the relevance of ($DiOC_6(3)$) as a quantitative marker of mΔΨ.

When mΔΨ was measured during SSP-induced apoptosis, a time-dependent reduction in the number of cells displaying high membrane potential was seen. However, when the fluorescence of the viable cells (those in the upper-right quadrant) was measured, there was no significant variation during the period of treatment. This may suggest that rather than being

a central event in the process of apoptosis, the observed reduction in mΔΨ was merely a consequence of the cells dying and therefore occurred late in the process. Pre-treatment with CsA caused a significant inhibition in the loss of mΔΨ implicating opening of the MPTP as the cause of the loss of potential. However, the inhibition observed was only slight, supporting the theory that induction of the MPTP is a non-essential, end-point event in this model of apoptosis. As CsA was able to produce a significant increase in mΔΨ of untreated cells, the possibility that it was directly interacting with ($DiOC_6(3)$) and influencing the staining must be considered. However, when the effect of BA was analysed, exactly the same results were obtained. This shows that both inhibitors successfully entered the cells and would suggest that they were able to influence mΔΨ by stabilising the MPTP as has been frequently reported. The slight inhibitory effect of BA also lends further weight to the theory that MPTP induction is not a crucial event of SSP-induced apoptosis in primary cardiomyocytes.

Measurement of mΔΨ following t-BOOH treatment revealed a significant decrease in both the number of cells displaying high membrane potential and in the mΔΨ of the remaining viable population. This suggests that loss of transmembrane potential could have been an important factor in necrotic cell death as the mitochondria were undergoing a reduction in potential prior to the cells undergoing a reduction in size. Pre-treatment with CsA resulted in a significant increase in the number of cells retaining high membrane potential following t-BOOH treatment and a highly significant increase in the mΔΨ of the remaining viable population. This showed that CsA was effectively inhibiting the reduction in transmembrane potential but due to the severity of the insult, it was only a transient inhibition.

When mΔΨ was analysed following 3hr of simulated ischaemia, no variation was observed. Upon simulated reperfusion, no change could be seen for up to 4hr. It was only when the cardiomyocytes were reperfused overnight for 20hr that a significant reduction in transmembrane potential was observed. As with the t-BOOH-treated cells, a significant loss of potential was observed in the mitochondria prior to the cells undergoing a reduction in size suggesting that loss of mΔΨ was a significant factor in the process of cell death. The fact that pre-treatment with CsA significantly inhibited the loss of potential implicated involvement of the MPTP. Pre-treatment with BA delivered the same results, confirming the involvement of the pore. However neither inhibitor was able to completely prevent the decline of mΔΨ. This could be due to a number of factors. Although the inhibitory effect of CsA and BA shows induction of the MPTP, there is no certainty that this accounts for the total decrease of mΔΨ. There are known to be other agents that cause disruption of the mitochondria (CCCP being an obvious example) and many other damaging factors present during reperfusion, so there may be other reasons for the decline in mΔΨ. The possibility that an element in the simulated ischaemic solution was directly affecting the transmembrane potential can probably be discounted, since no effect was seen during the period of application. By the time any decrease was observable the simulated ischaemia solution had been washed off and the cardiomyocytes had been incubating in maintenance media for 20hr making this an extremely unlikely scenario. It is also possible that induction of MPTP did account for the total decline in mΔΨ but that the inhibitors used were nonetheless able to exert only a partial influence. Other studies have reported complete inhibition by both of these agents (118, 149, 245, 246) but different cell types were used. Many different methods of inducing a loss of mΔΨ have also been applied to many different cells types so it is virtually impossible to judge the severity of the insult. If more convergent conditions or cell types had been used in these studies, results in closer

agreement might have been obtained. Additionally, it has been demonstrated that under severe enough conditions such as high Ca^{2+} concentration, the inhibitory effect of CsA may be overcome allowing induction of the MPTP (151, 187-189). It is also possible that the inhibitors were unable to bind with sufficient affinity or that they became detached during the course of the experiment so that their influence was only transient.

4.6 Conclusions

In conclusion, a significant decline in mΔΨ could be seen in all 3 models of cell death. Inhibition of the MPTP produced a significant effect in all instances proving the involvement of the pore. However in the case of the model of SSP-induced apoptosis, induction of the MPTP did not appear to be playing a very significant role. In all models complete inhibition by CsA or BA was never observed showing that other factors were influencing the outcome.

5: Expression and Activity of Cyclophilin D during Cardiomyocyte Death

5.1 Introduction

Peptide bonds within proteins can exist in both a *cis* and a *trans* form. During protein folding and refolding, the rate of reaction is frequently determined by the time required for the relevant peptide bond to shift from the *cis* to the *trans* state. Proteins known as peptidyl prolyl *cis-trans*-isomerases catalyse this shift thus speeding up the rate-limiting reaction. There are 3 unrelated families of proteins displaying peptidyl prolyl *cis-trans*-isomerase (PPI-ase) activity, the FK506 binding proteins (FKBPs) and the cyclophilins (CyP) (reviewed in 169) and the parvulins (168). The cyclophilin family as found in mammalian tissues consists of cyclophilin A, an 18kDa protein found in the cytoplasm, cyclophilin B and C (21kDa) found in the endoplasmic reticulum, and cyclophilin D (CyPD) an 18kDa protein localised to the mitochondria (162-165). Besides their PPI-ase activity, the cyclophilin family display endonuclease activity (166) and are stress-inducible (174). They bind cyclosporin A (CsA) and have been found in larger protein complexes such as the oestrogen receptor, (172) the nuclear pore complex (173) and the mitochondrial permeability transition pore (MPTP).

Many groups have now published evidence of the interaction of CyPD with the MPTP (140, 159, 161, 247). Much of this evidence is based on the effect of CsA on the pore since it is well known that CsA prevents the binding of CyPD to the inner mitochondrial membrane. One proposed mechanism for the opening of the MPTP is through the binding of CyPD to adenine nucleotide translocase (ANT), sensitising it to a calcium ion-induced conformational change (157). According to this theory, the interaction with CsA would prevent CyPD from binding to ANT and would render the pore less sensitive to the presence of calcium.

It is therefore possible that CyPD could be targeted therapeutically in order to prevent pathological induction of the MPTP.

5.2 Objective

The aim of this chapter was to determine whether CyPD was undergoing any changes in activity or expression during cell death.

It was also intended to establish whether CsA was successfully binding to and inhibiting the activity of CyPD.

5.3 Materials and Methods

Neonatal primary cardiomyocytes were isolated and cultured as described in chapter 2.2.1. Cell death was induced using staurosporine (SSP), t-butylhydroperoxide (t-BOOH) and a solution designed to replicate the extracellular conditions of ischaemia-reperfusion injury as detailed in chapter 2.2.2-4.

The mitochondrial fraction of the primary cardiomyocytes was harvested according to a standard method of centrifugation as described in chapter 2.5.1.

The PPI-ase activity of the mitochondrial sample was measured according to the kinetic method adapted by Kofron as detailed in chapter 2.5.2.

A modification of the peptide sequence used by Bergsma and co-workers was used to raise antibodies to rat CyPD in rabbits. These were then purified and utilised in a standard Western blotting procedure. For details see chapter 2.5.3 and 2.8.1-2.

5.4 Results

5.4.1 Measurement of PPI-ase activity

PPI-ase activity was quantified by measuring the ability of cyclophilins to catalyse the cleavage of a cyclophilin-specific peptide by shifting peptide bonds from a *cis* to a *trans* form. When cyclophilin A at concentrations ranging from 5-30nM was added to the basic assay (8mg/ml α-chymotrypsin in 35mM HEPES buffer with 8mM peptide), a concentration-dependent increase in the rate of reaction was observed (Figure 45a). The rate of reaction was computed and the CyP-induced increase in rate was expressed as the percentage increase over the rate of the control assay (Figure 45b).

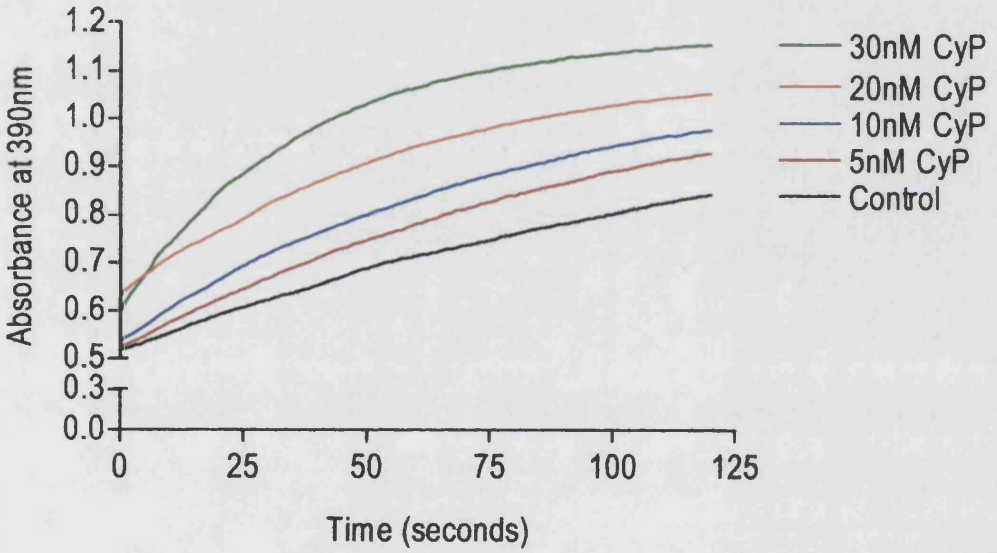


Figure 45a: Cyclophilin (CyP) was added to the control reaction at a concentration range of 5-30nM and the absorbance at 390nm was recorded every 0.25 sec for 2 min. This representative graph shows the ability of cyclophilin A to catalyse the conversion of the peptide in a concentration dependent manner.

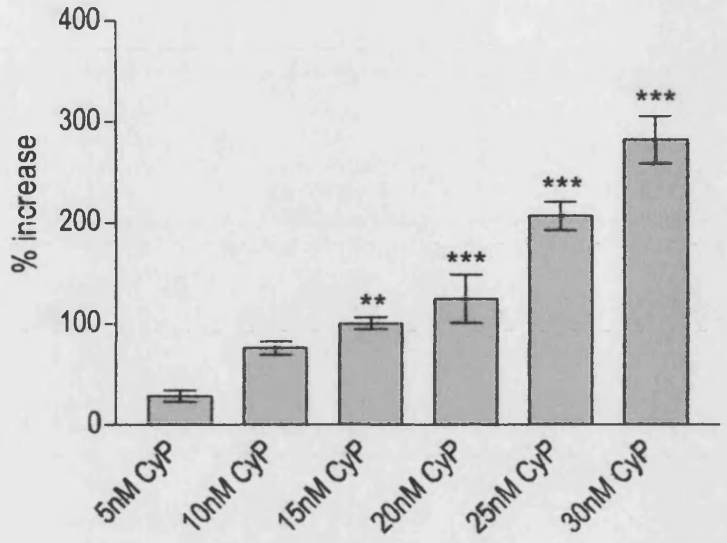


Figure 45b: The increase in the reaction rate was calculated and expressed as the percentage increase over the basic reaction for statistical analysis, showing a significant increase from a cyclophilin (CyP) concentration of 15nM [**, $p < 0.01$; ***, $p < 0.001$].

To test the ability of CsA to inhibit the PPI-ase activity of CyP in this assay, CsA at concentrations ranging from 1nM - 5μM was added to a reaction also containing 30nM CyPA. This clearly demonstrated that CsA was an extremely potent inhibitor of the CyP-induced increase in the reaction rate at all the concentrations tested, demonstrating that the ability of CyP to increase the rate of reaction was due to its PPI-ase activity (see Figure 46a).

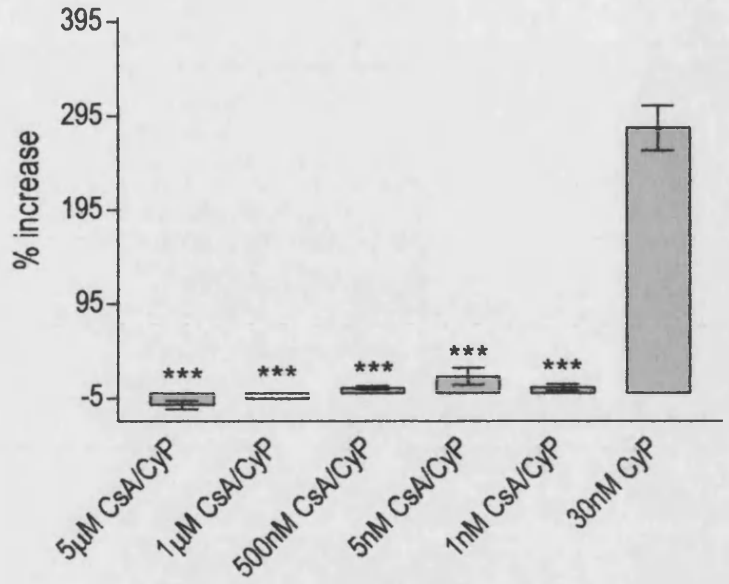


Figure 46a: When added to an assay containing 30nM cyclophilin (CyP), cyclosporin A (CsA) significantly inhibited the CyP-induced increase in the reaction rate at concentrations ranging from 1nM - 5μM [***, $p < 0.001$ v. 30nM CyP].

FK506-binding proteins are well-established possessors of PPI-ase activity in the cell. To show that these were not affecting the reaction, FKBP was added to the basic assay at concentrations ranging from 250nM - 5μM. This demonstrated that FKBP had a non-significant catalytic effect compared to cyclophilin as shown in Figure 46b.

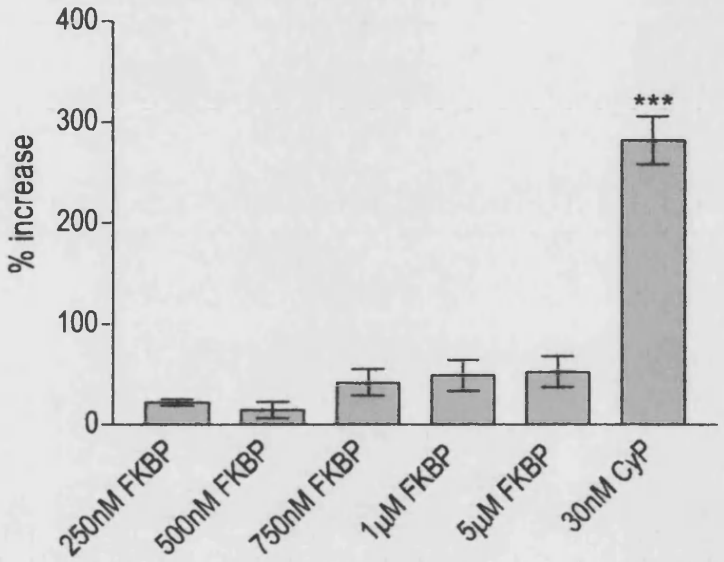


Figure 46b: When FK506-binding protein (FKBP) was added to the basic assay, analysis of reaction rates showed that it was unable to significantly increase the rate of reaction in comparison to 30nM cyclophilin (CyP) [***, $p<0.001$ v. basic assay].

5.4.2 Mitochondrial PPI-ase activity in apoptotic primary cardiomyocytes

The data presented above shows that the assay of increased rate of peptide cleavage is a suitable method for the quantification of CyP PPI-ase activity. Therefore, it was employed for the measurement of CyP PPI-ase activity in neonatal primary cardiomyocytes as detailed below.

The mitochondrial fraction was isolated from primary cardiomyocytes by a standard method of differential centrifugation to produce a preparation of pure mitochondria with normal ultrastructure.

Isolation of the mitochondrial fraction and quantification of the PPI-ase activity was utilised to directly examine the activity of CyPD following treatment with the apoptosis inducer staurosporine (SSP). No change in PPI-ase activity could be seen in the SSP-treated samples. However, pre-treatment with CsA significantly reduced PPI-ase activity as shown in Figure 47.

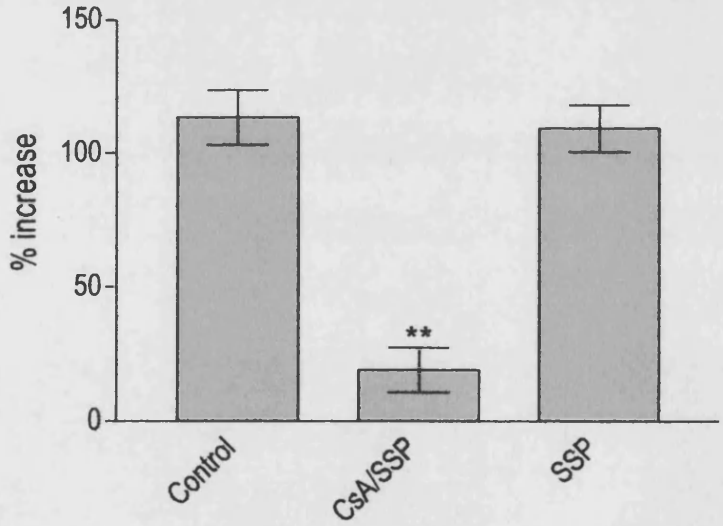


Figure 47: The cyclophilin PPI-ase enzyme activity of the cardiomyocyte mitochondrial fraction was assayed over 2 min and expressed as the percentage increase over the assay without mitochondrial fraction. No change in activity was observed after 20hr of 5μM staurosporine

(SSP) treatment but 1hr pre-treatment with 50nM cyclosporin A (CsA) produced significant inhibition of PPI-ase activity [**, $p < 0.01$ v. control].

5.4.3 Mitochondrial PPI-ase activity in necrotic primary cardiomyocytes

Primary cardiomyocytes were treated with t-BOOH for 1hr prior to isolation of the mitochondrial fraction and analysis of the PPI-ase activity. No change in PPI-ase activity could be seen following necrotic cell death. However, pre-treatment with CsA significantly reduced PPI-ase activity as shown in Figure 48.

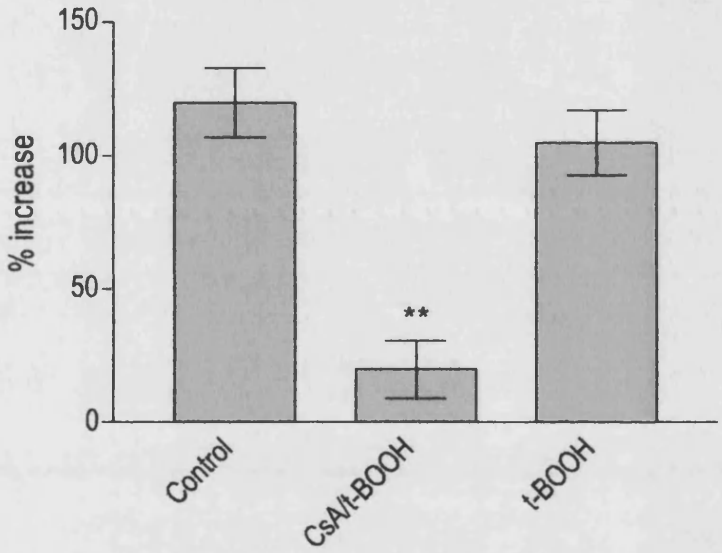


Figure 48: The cyclophilin PPI-ase enzyme activity of the cardiomyocyte mitochondrial fraction was assayed over 2 min and expressed as the percentage increase over the reaction without mitochondrial fraction. No change in activity was observed after 1hr of 100μM t-butylhydroperoxide (t-BOOB) treatment but 1hr pre-treatment with 50nM cyclosporin A (CsA) produced significant inhibition of PPI-ase activity [**, $p<0.01$ v. control].

5.4.4 Mitochondrial PPI-ase activity following simulated ischaemia-reperfusion injury

Primary cardiomyocytes were subjected to simulated ischaemia-reperfusion injury (according to Chapter 2.2.4) prior to isolation of the mitochondrial fraction and analysis of the PPI-ase activity. Although no change in activity was detectable, pre-treatment with CsA significantly reduced PPI-ase activity as shown in Figure 49.

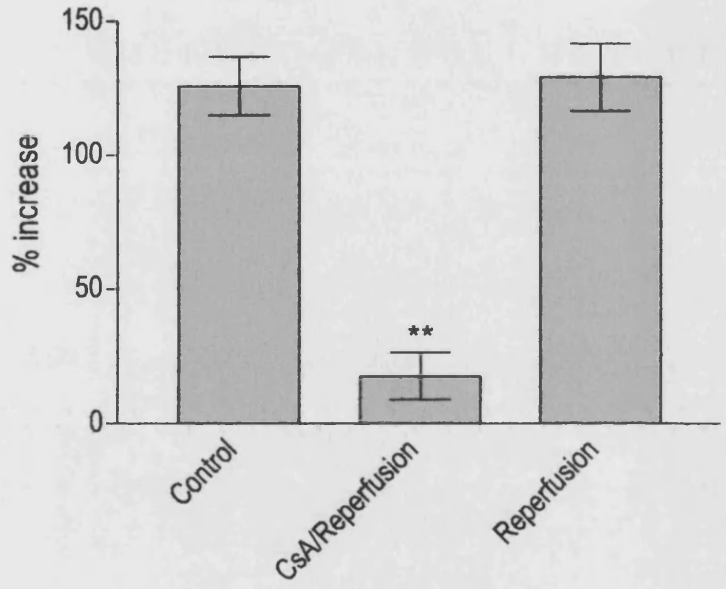


Figure 49: The cyclophilin PPI-ase enzyme activity of the cardiomyocyte mitochondrial fraction was assayed over 2 min and expressed as the percentage increase over the reaction without mitochondrial fraction. No change in activity was observed following 3hr of simulated ischaemia and 20hr simulated reperfusion but 1hr pre-treatment with 50nM cyclosporin A (CsA) produced significant inhibition of PPI-ase activity [**, $p < 0.01$ v. control].

5.4.5 Detection of Cyclophilin D protein in primary cardiomyocytes

A specific antibody to CyPD was raised in rabbits according to the method of Bergsma and colleagues, and purified from whole serum as described in chapter 2.5.3. The efficiency of the purification was analysed by an ELISA detection of CyPD antibody. Comparison of CyPD

antibody content of the purified antibody to both the original serum and the serum after affinity column binding showed that successful purification had been achieved (Figure 50).

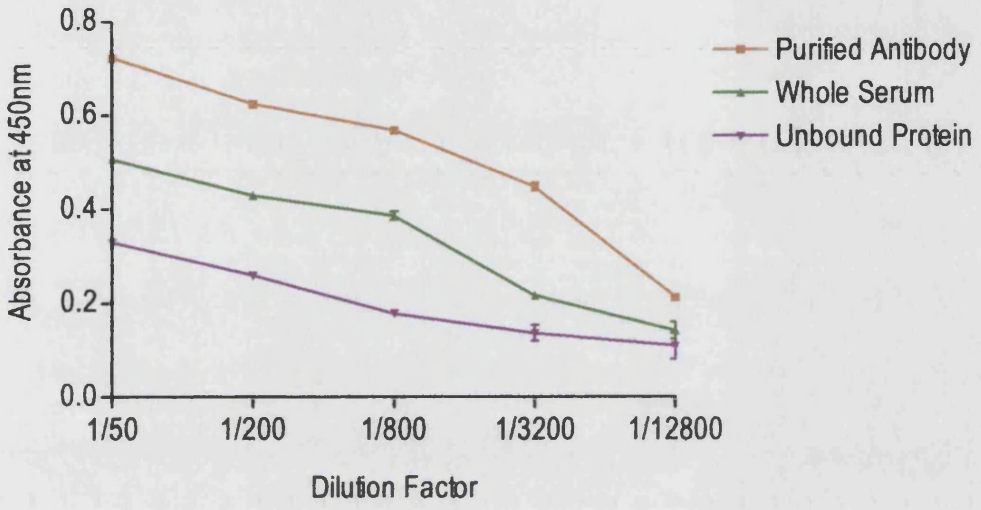


Figure 50: Cyclophilin D (CyPD) antibodies were detected using an ELISA assay. Comparison of antibody content in whole rabbit serum, serum after affinity column binding and purified antibody showed that the CyPD antibodies had been successfully isolated.

The purified antibody had a concentration of 15µg/ml and was used to detect CyPD in whole primary cardiomyocytes cell lysate by a standard Western blot protocol. The results obtained were extremely small bands at the limit of visibility but there was no obvious variation in CyPD following simulated ischaemia-reperfusion injury (Figure 51).

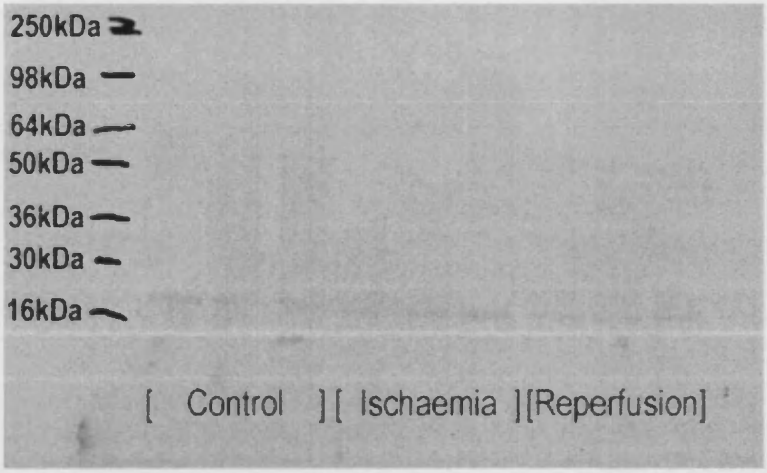


Figure 51: Primary cardiomyocytes were subjected to 3hr of simulated ischaemia followed by 20hr of simulated reperfusion and the cell lysates were probed with an antibody specific to cyclophilin D (CyPD). Although faint protein bands were seen at ~18kDa, no clear variation in expression was observed.

5.5 Discussion

CyPD is located in the mitochondrial matrix and its binding to the ANT on the inner mitochondrial membrane is believed to be an important part of MPTP induction. Analysis of the cyclophilin/CsA complex has shown that CsA physically blocks the cyclophilin site of PPI-ase activity (206). It is thought by some groups that this prevents CyPD from binding to the ANT thereby preventing formation of the pore complex.

In order to examine the activity of CyPD in the primary cardiomyocytes, the PPI-ase activity mitochondrial fraction was measured. No change in activity was seen in any of the models of cell death. However, pre-treatment with CsA resulted in a significant inhibition of PPI-ase activity in all instances. This shows that CsA was successfully entering the mitochondria and binding to the active site of CyPD for the duration of the experimental procedures.

Although the effect of CsA on PPI-ase activity was highly significant, complete inhibition was never observed. The concentration of CsA selected was the highest concentration that showed absolutely no toxic effect (see chapter 3.5.4), but it is possible that this was too little to bind to and inhibit all of the CyPD present.

Cyclophilin-like proteins and other molecules with PPI-ase activity are continually being discovered (180-182). A second possibility therefore, is that another as yet unidentified protein in the mitochondrial fraction was contributing to the detectable PPI-ase activity. However as the peptide used in the assay has extremely high affinity for cyclophilins and it has been demonstrated that CyPD is the only mitochondrial target of CsA (140), this option is not very probable.

A more likely possibility is that the presence of CsA resulted in a compensatory increase in CyPD production in the cardiomyocytes as a recent study has shown in human cancer cell lines (248).

Western blotting with a specific CyPD antibody was intended to resolve this issue. The antibody in question was developed by Bergsma and colleagues and reported in a paper published in 1991 (162). In their paper the group showed specificity of the antibody and demonstrated that it did not cross-react with the other cyclophilins. Unfortunately the relevant protocols could not be replicated for this study, as only the CyPA protein is currently commercially available. However, Western blotting with the purified antibody revealed a protein band, which although faint, did appear at the correct position for CyPD.

This procedure was ultimately performed only on the model of ischaemia-reperfusion injury due to the huge number of cells required to obtain visible protein bands. Isolation of the mitochondrial fraction would have reduced the chances of cross-reaction with other cyclophilins and possibly improved the resolution of the CyPD protein bands. However, this proved unfeasible, as the amount of mitochondrial protein obtainable from the primary cardiomyocytes was too little to be successfully detected by Western blotting. Therefore whole cell lysates were used.

Although the protein bands resolved were too small and faint to effectively quantify, the PPI-ase activity seen in the CsA-treated samples could be accounted for by a protein increase of no more than 5-10% and would probably be difficult to detect by a technique such as Western blotting.

5.6 Conclusions

The PPI-ase activity of CyPD was unaffected by the application of inducers of apoptosis and necrosis, or by the *in vitro* simulation of ischaemia-reperfusion injury. However pre-treatment with CsA produced a significant decrease in activity, showing that CsA enters the mitochondria and remains bound to CyPD for the duration of these experiments.

6: ATP Production and Free Radical Activity: The Effect of MPTP Inhibition

6.1 Introduction

As inhibitors of the mitochondrial permeability transition pore (MPTP) had proved unable to exert more than a minimal influence over the progression of cell death in primary cardiomyocytes, it was assumed that other factors were affecting the ability of the cells to survive the insults.

Generation of ATP is one of the most crucial functions of the mitochondria, providing the cell with the energy that it needs to sustain itself. It has for some time been believed that the levels of ATP in the mitochondria can exert a major influence on the progression of cell death. Studies have suggested that the availability of ATP may act as an "intracellular switch" determining whether the cell proceeds to die through apoptosis or necrosis (61-63).

Another parameter of cellular injury that is frequently studied is the generation of free radicals. Increased free radical activity has been demonstrated during both ischaemia and reperfusion and many potential sources of free radical production within the cell have been proposed (reviewed in 249). The respiratory chain in the mitochondrial membranes has been proposed as a major source of free radical production during ischaemia-reperfusion injury. This site of injury is particularly relevant to this study due to the large number of mitochondria present in cardiomyocytes and the potential for MPTP induction to seriously affect the respiratory chain. Additionally, free radicals can destabilise lipid membranes and damage DNA, so the extent of their production in the cell may be an important factor in determining whether the cell will suffer irreversible damage.

6.2 Objective

The aim of this study was to examine other important aspects of cell death and determine whether these were playing a major role in the primary cardiomyocytes.

The study aimed to quantify the production of ATP and the generation of free radicals during apoptosis, necrosis and simulated ischaemia-reperfusion injury and to determine whether inhibitors of the MPTP would affect these parameters.

6.3 Materials & Methods

Neonatal primary cardiomyocytes were isolated and cultured as described in chapter 2.2.1.

Cell death was induced using staurosporine (SSP), t-butylhydroperoxide (t-BOOH) and a solution designed to replicate the extracellular conditions of ischaemia-reperfusion injury as detailed in chapter 2.2.2-4.

The ATP content of primary cardiomyocytes was measured using a standard bioluminescence assay (see Chapter 2.6).

Free radical production in primary cardiomyocytes was measured using the free radical sensitive fluorescent dye 2',7'-dichlorodihydrofluoroscein diacetate (H_2DCFDA) (see Chapter 2.7).

6.4 Results

6.4.1 Measurement of ATP in primary cardiomyocytes

The most reliable means of calculating ATP release from the samples was to include an internal standard in the bioluminescence based assay. Therefore, the ATP release from a sample of primary cardiomyocytes was measured and compared to the light emission data obtained when the sample was combined with a known range (1ng-10μg/ml) of ATP standards. The commercial assay used required an internal standard that produced a reading approximately double that of the sample alone (see chapter 2.6), a criterion that was met by the combination of 50μl of 5μg/ml ATP and a 50μl sample of cell suspension (Figure 52a). Therefore, 5μg/ml ATP was selected as an internal standard in all experiments in order to calculate the ATP content of each sample. Addition of cells in suspension and the kit component Somatic Cell ATP Releasing Reagent caused significant quenching as shown in Figure 52b, but as this was consistent throughout it did not alter the effectiveness of the assay. It was also noted that the emission of light rapidly reduced following initiation of the reaction (Figure 52c). However, this did not affect the results obtained as each sample was measured along with an internal standard ensuring that the ATP value could be accurately calculated in each instance, despite the declining light emission.

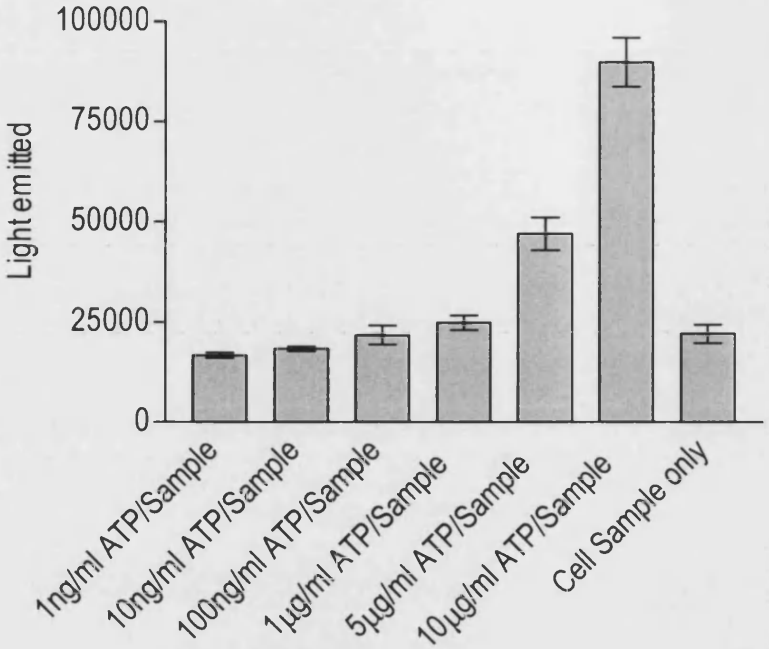


Figure 52a: The ATP released by a control sample of cardiomyocytes in suspension was measured with a range of ATP standards from 1ng-10µg/ml. 5µg/ml ATP was chosen for use as an internal control as it gave a reading of approximately double that of the sample alone.

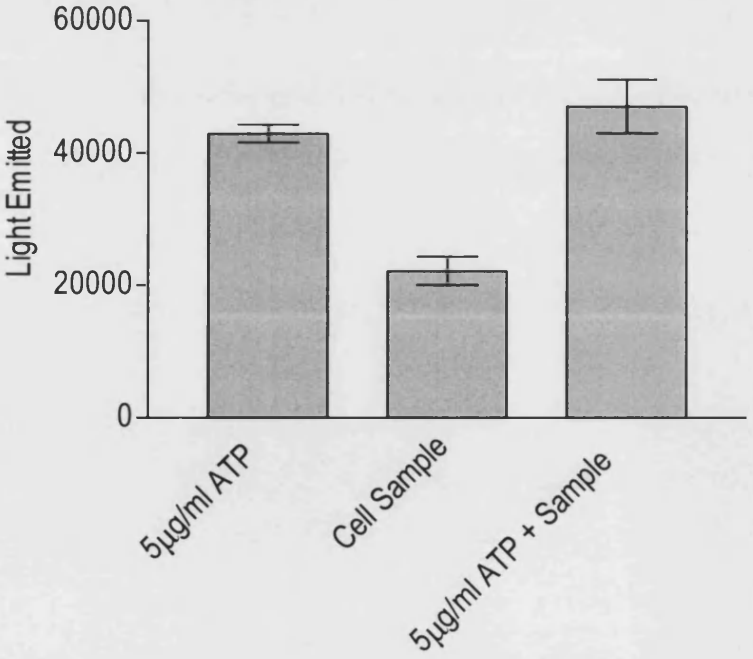


Figure 52b: Addition of cells in suspension and Somatic Cell ATP Releasing Reagent caused quenching of emitted light relative to the ATP standards (i.e. the combination of ATP and cell

sample does not produce the same emitted light as the sum of the two separate readings).

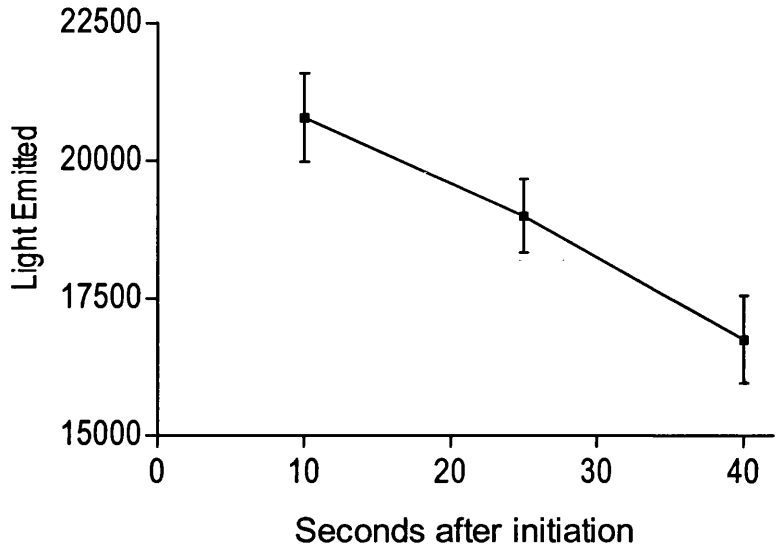


Figure 52c: The amount of light emitted by a sample of cells in suspension began to decrease in a linear fashion immediately following initiation of the reaction.

6.4.2 The effect of CsA on ATP content during apoptosis

Primary cardiomyocytes were treated with 50nM CsA for 1hr prior to SSP application for 20hr. Analysis of ATP content showed a significant decrease in ATP that was not inhibited by CsA (Figure 53).

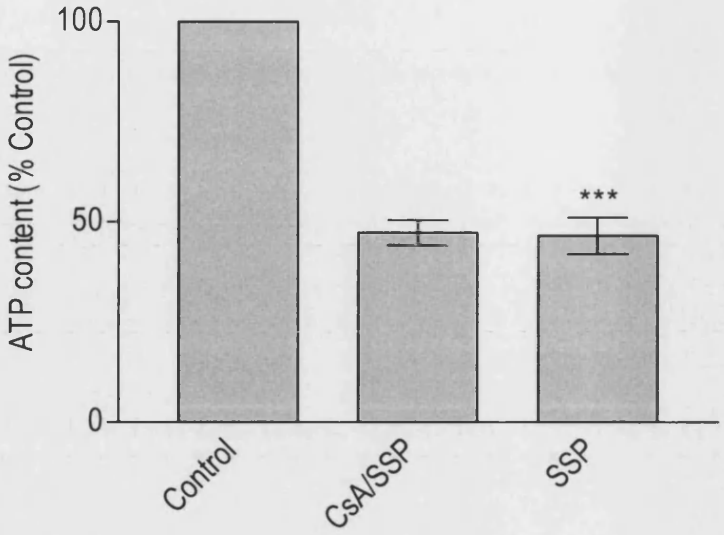


Figure 53: Compared to the control cells (ATP = 0.94µg/ml, SD = 0.23) the ATP content of the cardiomyocytes was reduced after 20hr of 5µM staurosporine (SSP) application but pre-treatment with 50nM cyclosporin A (CsA) did not show any significant influence [***, $p < 0.001$ v. control].

6.4.2 The effect of CsA on ATP content during necrosis

50nM CsA was applied to primary cardiomyocytes prior to treatment with 100µM t-butylhydroperoxide and analysis of ATP content. The onset of necrosis resulted in the reduction of ATP content to virtually nothing. Pre-treatment with CsA resulted in a very slight, although significant inhibition (Figure 54).

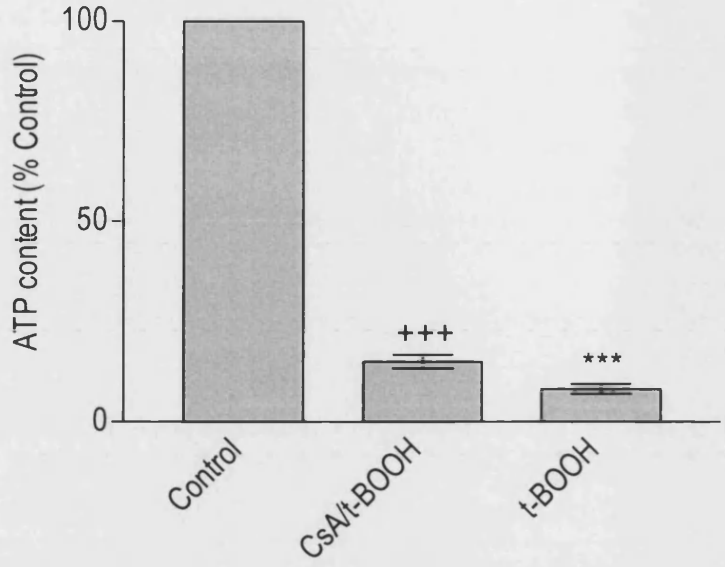


Figure 54: Compared to the control cells (ATP = 0.76µg/ml, SD = 0.04), the ATP content of the cardiomyocytes was greatly reduced 1hr after application of 100µM t-butylhydroperoxide (t-BOOH) but pre-treatment with 50nM cyclosporin A (CsA) showed only a small, albeit significant influence [***, $p < 0.001$ v. control; +++, $p < 0.001$ v. t-BOOH].

6.4.3 The effect of CsA on ATP content during simulated ischaemia-reperfusion injury

50nM CsA was applied to primary cardiomyocytes for 1hr prior to subjection to simulated ischaemia-reperfusion injury. ATP content was analysed following the 3hr simulated ischaemia period and also after 20hr simulated reperfusion. Following 3hr simulated ischaemia the ATP content of the cells was greatly reduced but after the period of simulated reperfusion this had recovered an appreciable amount although the ATP content was still significantly less than the control cells. CsA pre-treatment did not show any significant influence on ATP during either simulated ischaemia or simulated reperfusion although a slight insignificant inhibition was reliably observed following reperfusion (Figure 55).

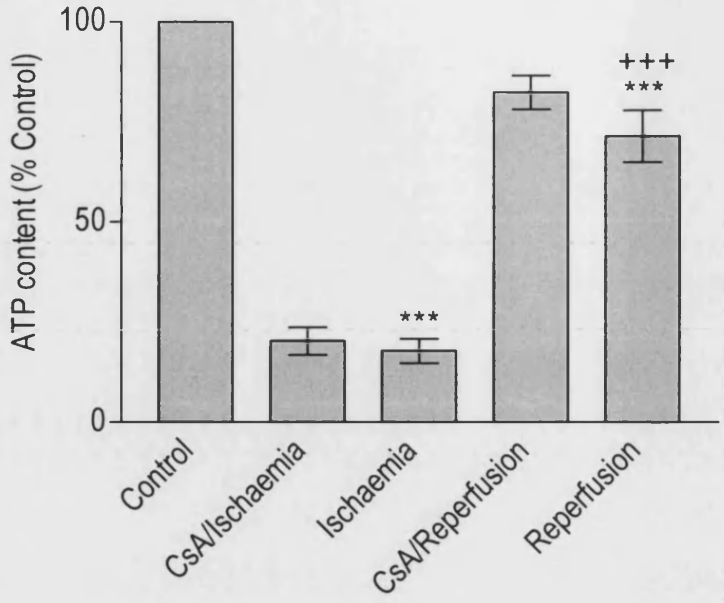


Figure 55: Compared to the control cells (ATP = 0.935μg/ml, SD = 0.10), 3hr of simulated ischaemia resulted in a large decrease in ATP content that was not inhibited by cyclosporin A (CsA) pre-treatment. After 20hr simulated reperfusion much of the ATP content was recovered and was again not significantly influenced by CsA pre-treatment [***, $p < 0.001$ v. Control; +++, $p < 0.001$ v. Ischaemia].

6.4.4 Toxicity of 2',7'-dichlorodihydrofluoroscein diacetate in primary cardiomyocytes

The free radical sensitive dye 2',7'-dichlorodihydrofluoroscein diacetate (H_2DCFDA) was applied to primary cardiomyocytes at a wide range of concentrations designed to determine toxicity (500nM-5μM). Analysis of LDH release showed a slight increase at 3μM with significant toxicity at 5μM as shown in Figure 56.

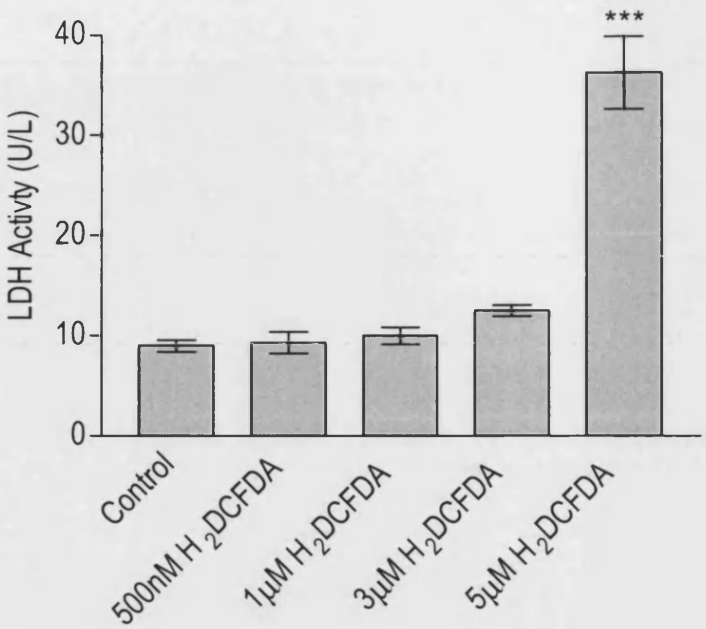


Figure 56: H_2DCFDA attained significant toxicity to primary cardiomyocytes from 5μM as determined by release of LDH [***, $p < 0.001$ v. Control].

6.4.5 The effect of CsA on free radical activity during apoptosis

Primary cardiomyocytes were treated with 50nM CsA before 500nM H_2DCFDA was added to the media simultaneously with 5μM SSP. The cells were then incubated in the dark for 20hr prior to measurement of fluorescence on a flow cytometer. A negative control was initially analysed and in subsequent samples, the number of cells displaying fluorescence greater than the parameters of the negative control were counted. Analysis showed that all samples displayed an extremely low level of free radical activity with no discernible difference between control and apoptotic samples (Figure 57).

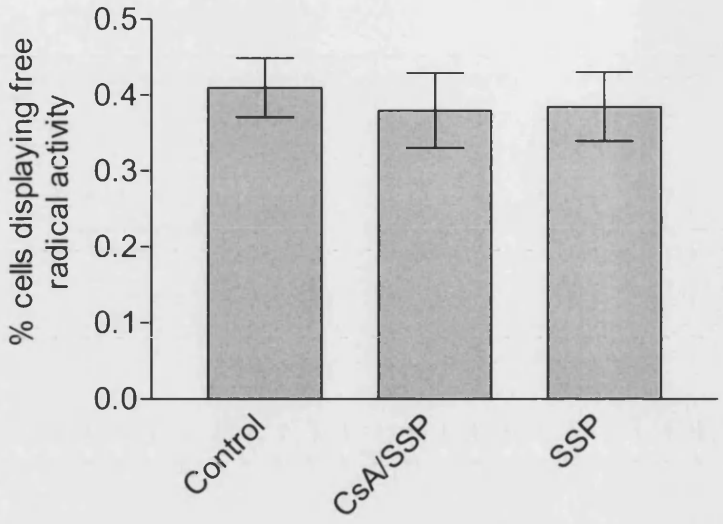


Figure 57: Neither 24hr of 5μM staurosporine (SSP) treatment nor 1hr pre-application of 50nM cyclosporin A (CsA) had any effect on the generation of free radicals in primary cardiomyocytes (determined by free radical conversion of H_2DCF to fluorescent form).

6.4.6 The effect of CsA on free radical activity during necrosis

Primary cardiomyocytes were treated with 50nM CsA before 500nM H_2DCFDA was added to the media simultaneously with t-BOOH. The cells were then incubated in the dark for 1hr prior to measurement of fluorescence on a flow cytometer. Analysis showed that there was a highly significant increase in cells displaying free radical activity following t-BOOH treatment. Pre-treatment with CsA did not influence the level of activity (see Figure 58).

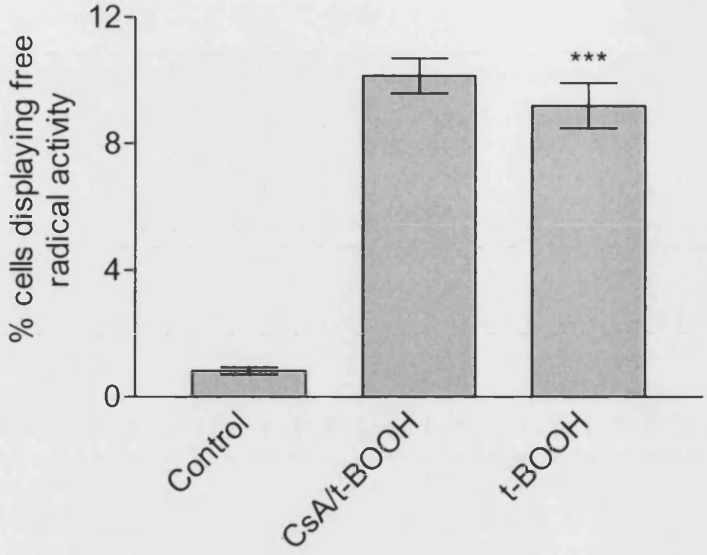


Figure 58: Treatment of primary cardiomyocytes with t-butylhydroperoxide (t-BOOH) for 1hr resulted in a highly significant increase in free radical activity (determined by free radical conversion of H_2DCF to fluorescent form) that was unaffected by cyclosporin A (CsA) [***, $p < 0.001$ v. Control].

6.4.7 The effect of CsA on free radical activity during simulated ischaemia-reperfusion

Primary cardiomyocytes were treated with 50nM CsA prior to subjection to simulated ischaemia and reperfusion in the dark. If the cardiomyocytes were subjected to simulated ischaemia only, then 500nM H_2DCFDA was added to both samples and controls for the duration of the simulated ischaemia. If the cardiomyocytes were subjected to a subsequent period of simulated reperfusion, then H_2DCFDA was added to samples and controls during the simulated reperfusion period only. Following treatment, the fluorescence of all samples was measured on a flow cytometer. Analysis showed a significant increase in cells displaying free radical activity following simulated ischaemia. However, pre-treatment with CsA did not inhibit the level of activity and appeared to cause a slight increase in comparison with simulated

ischaemia although this was not significant. Following simulated ischaemia and reperfusion, there was a highly significant increase in free radical activity which was unaffected by CsA (see Figure 59).

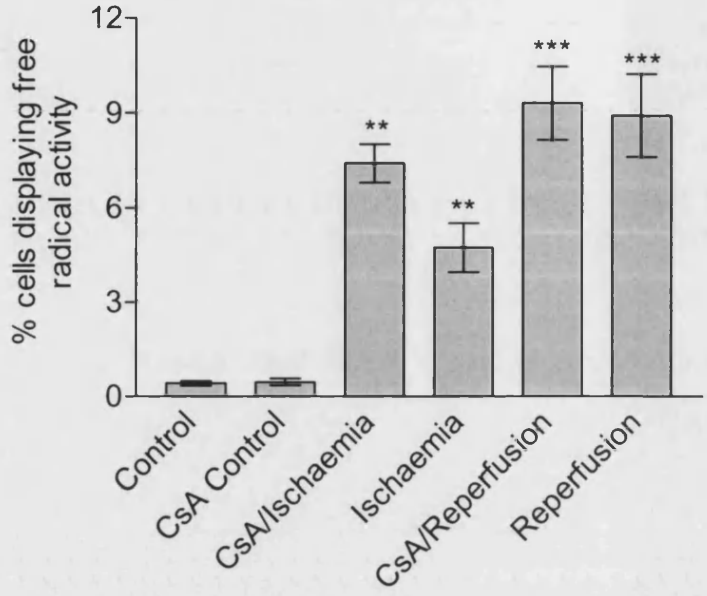


Figure 59: Primary cardiomyocytes subjected to 3hr of simulated ischaemia displayed a significant increase in free radical activity (determined by free radical conversion of H_2DCF to fluorescent form) that was not inhibited by pre-treatment with 50nM cyclosporin A (CsA). 20hr of simulated reperfusion resulted in a significantly raised level of activity that was unaffected by CsA [**, $p < 0.01$ v. Control; ***, $p < 0.001$ v. Control].

6.5 Discussion

The production of ATP by the respiratory chain is an essential process in all mammalian cells. ATP powers some of the most important intracellular reactions and is therefore essential to the survival of the cell. In the absence of oxygen, ATP production can continue for a time through glycolysis, but even this is eventually inhibited by the conditions present during ischaemia (74). The transfer of electrons across the virtually impermeable inner mitochondrial membrane by the complexes of the respiratory chain creates an electrochemical gradient, which drives ATP synthesis. Opening of the MPTP destroys this gradient, thus halting production of ATP and threatening the survival of the cell.

Apoptotic cell death is an active process that requires ATP to go to completion. Analysis of ATP levels in the primary cardiomyocytes following application of the apoptosis inducer SSP revealed a decrease of around 50%. This drop may represent cardiomyocytes in the post-apoptotic stage where all energy production has ceased. Pre-treatment with CsA did not influence the levels of ATP after 20hr, suggesting that inhibition of the MPTP has only a transient effect on ATP production, or no effect at all.

Induction of necrosis in cardiomyocytes caused an almost complete abolition of ATP production. Despite this, CsA pre-treatment slightly, but significantly inhibited this decline suggesting inhibition of the MPTP could have helped to preserve ATP production. However, levels of ATP were extremely low even in the CsA treated cells suggesting that the severity of the insult was able to overcome the inhibition resulting in only a partial inhibition. ATP production may have also declined due to direct inhibition of the respiratory chain that was unrelated to MPTP induction.

Inhibition of the respiratory chain during ischaemia-reperfusion injury has been demonstrated in many studies, predominantly the inhibition of respiration in complex I, which has been

demonstrated up to 2 hr after reperfusion (250). Following 3hr of simulated ischaemia in the primary cardiomyocytes, levels of ATP were extremely low. The fact that CsA was unable to preserve ATP production was fully expected as it has been shown on many occasions that the MPTP does not open during ischaemia.

After 20hr of simulated reperfusion, ATP production had significantly recovered although it was still significantly lower than in the control cardiomyocytes. CsA did not have a significant effect on recovery although a slight positive effect was consistently observed. Although many studies have shown direct inhibition of the respiratory chain complexes during ischaemia-reperfusion, the length of simulated reperfusion in this study probably allowed complete recovery. Therefore, the most probable explanation is that the majority of cell death occurring was not related to MPTP induction. The slight inhibition that was observed probably accounted for a sub-population of cells that were dying due to the induction of MPTP. This may again indicate that in this specific model, MPTP is largely a consequence of cell death rather than a cause.

It was noted that addition of cell suspension to the ATP assay caused some quenching of the light emitted. However, this level of quenching was consistent throughout so did not affect the results of the experiment since an internal control was present, and the intent was to examine the variation between samples rather than to obtain an accurate quantification of ATP in primary cardiomyocytes.

The generation of free radicals has been identified as a major factor in ischaemia-reperfusion injury with a number of studies also showing the protective effect of various free radical scavengers (108, 110, 111). The mitochondrial respiratory chain has been pinpointed as a major source of free radical production during ischaemia-reperfusion injury (93, 250, 251). As cardiomyocytes have a comparatively large quantity of mitochondria it is anticipated that they may be the major source of free radical production during cardiac ischaemia-reperfusion injury.

Following induction of apoptosis in primary cardiomyocytes for 20hr, no increase in free radical activity was observed. This result is as expected as it is known that SSP induces apoptosis without causing production of free radicals.

The inability of CsA to inhibit the increase in free radical production during necrosis shows that free radical generation most likely occurs throughout the cell, independently of MPTP induction. This may be due in part to direct generation of free radicals by the oxidant chemical t-BOOH. Although t-BOOH application did not appear to result in the large-scale generation of free radicals, it should be noted that the acetate group is cleaved from H_2DCFDA inside the cell and the product is largely sensitive to hydrogen peroxide (H_2O_2) and hydroxyl radicals (100). It is therefore possible that application of t-BOOH resulted in large-scale production of superoxide radicals that could directly cause damage to the cells. However, the fact that no effect of CsA was observed shows that free radical production is not significantly linked to MPTP induction.

Simulated ischaemia produced a significant increase in free radical production in the cardiomyocytes. However, when they were pre-treated with CsA, a slight although non-significant increase in free radicals over ischaemic cells was observed. As there was no change in activity when the cells were treated with CsA alone, the most likely explanation is that CsA was interacting with elements of the ischaemic solution. This issue could be settled using an alternative MPTP inhibitor. However, cell viability results obtained using the adenine nucleotide transporter inhibitor bongkrekic acid (BA) in this system were extremely erratic (see Chapter 3) and other MPTP inhibitors are not as readily available.

Many studies have shown an accumulation of superoxide radicals during ischaemia followed by a burst of hydroxyl radicals upon reperfusion that are proposed to originate from the degradation of superoxide radicals (reviewed in 249). The dye used in this study is known to detect hydroxyl radicals and H_2O_2 whilst being largely insensitive to superoxide radicals. The

fact that significant production of free radicals was observed during ischaemia is probably due to the fact that analysis by flow cytometry required the cardiomyocytes to be suspended in PBS. Although the time between harvesting and analysing the cells was as short as possible, some degradation of superoxide to H_2O_2 and hydroxyl radicals may have been encouraged. Following simulated reperfusion of the primary cardiomyocytes, there was a highly significant increase in free radical production that was not inhibited by CsA. This production is a cumulative effect over 20hr that does not reveal the period of greatest free radical generation, as the intent was to monitor the effect of CsA following a long period of simulated reperfusion. The inability of CsA to affect the generation of free radicals shows that they are produced independently of MPTP induction. Xanthine oxidase is a possible source of free radicals in this model but significantly greater contribution from the respiratory chain would be expected due to the large number of mitochondria present in cardiomyocytes. During ischaemia the respiratory chain complexes become highly reduced leading to donation of electrons to residual oxygen molecules, thus forming superoxide radicals. Upon reperfusion, these are converted to the even more damaging hydroxyl radicals for which, unlike superoxide and H_2O_2, the cell does not produce any dedicated enzymatic scavengers. As the chain of events is initiated during ischaemia, and MPTP induction is only observed upon reperfusion, CsA would not be expected to influence free radical production by the respiratory chain complexes. As CsA had no discernable effect on the generation of free radicals, this shows that all production of free radicals in this model is independent of MPTP induction. This is consistent with current theories of free radical production during ischaemia-reperfusion injury. This also means that inhibiting the MPTP in an *in vivo* situation would not attenuate free radical mediated damage.

Free radical production during ischaemia and reperfusion cannot be directly compared due to the differing application times of H_2DCFDA. However, the intent in this instance was to

determine the effect of CsA application in each case. H_2DCFDA is a widely used dye that was ideal for this study as it is sensitive to H_2O_2 and hydroxyl radicals, which are generated during reperfusion when the MPTP is presumed to open. The free radical-sensitive dye dihydrorhodamine 1,2,3 is known to localise to the mitochondria. However, this dye is related to rhodamine 1,2,3 which is utilised for measurements of $m\Delta\Psi$ so would probably not be a reliable probe in this instance of both energised and de-energised mitochondria.

6.6 Conclusions

In conclusion, apoptosis proceeded with no extra production of free radicals, but the failure of CsA to inhibit the decline in ATP production again implied that the MPTP was not playing a significant role.

During necrosis, CsA had a slight inhibitory effect on the decline of ATP production showing that lack of ATP was a contributing factor towards cell death. However, CsA had no influence over free radical production during necrosis suggesting that the majority of free radical production may be outside the confines of the mitochondria or unrelated to the respiratory chain.

CsA was unable to significantly effect the generation of free radicals during simulated ischaemia-reperfusion injury. This shows that inhibition of the MPTP cannot influence this parameter in this particular model. However, the failure of CsA to inhibit the decline in ATP production may suggest that the MPTP does not significantly contribute to cell death in this model of ischaemia-reperfusion injury.

7: The MPTP and CyPD in an *In Vivo* Model of Cardiac Ischaemia-Reperfusion Injury

7.1 Introduction

Although numerous studies have examined mitochondrial permeability transition pore (MPTP) induction in isolated cardiomyocytes, substantially less work has been done using intact cardiac tissue. The major difficulty with studies using intact myocardium is that the techniques commonly employed for measuring mitochondrial parameters *in vitro* cannot be easily adapted to an *in vivo* situation. This means that some question remains as to whether the process under examination is merely an *in vitro* artefact.

Studies by Halestrap and colleagues have examined the behaviour of the MPTP in isolated perfused hearts. This work has shown that the MPTP opens during reperfusion only (130) and that inhibition by cyclosporin A (CsA), results in increased survival of the heart (136). This immediately suggests a potential role for MPTP inhibiting drugs in situations such as cardiac operations.

However, no study has yet attempted to examine the MPTP in myocardium subjected to ischaemia-reperfusion injury *in situ*. Although there is no obvious method of analysing the MPTP *in vivo*, a significant decline in $m\Delta\Psi$ is frequently cited as a strong implication of MPTP induction.

Therefore the purpose of this chapter was to attempt to directly measure the $m\Delta\Psi$ of mitochondria isolated from rat hearts subjected to *in vivo* ischaemia and reperfusion. This could be a useful indicator as to whether opening of the MPTP is truly a pathological event. It was also intended to examine the activity of cyclophilin D (CyPD), which is usually studied only by indirect means such as the action of CsA.

7.2 Objective

The aim of this study was to attempt to examine the $m\Delta\Psi$ of mitochondria isolated from hearts subjected to *in vivo* ischaemia-reperfusion injury and to explore the potential action of CyPD.

7.3 Materials and Methods

A model of cardiac ischaemia-reperfusion was set up in adult Sprague-Dawley rats and was assessed morphologically and by plasma creatine kinase (CK) activity. The $m\Delta\Psi$ was analysed in subsequently isolated mitochondria using a TPP^+ sensitive electrode. For details please see chapter 2.10.

PPI-ase activity of the isolated mitochondrial fraction was measured by spectrophotometric assays as described in chapter 2.5.2 and CyPD protein was identified by Western blotting with a specific CyPD antibody as detailed in 2.8 and 2.5.3 respectively.

7.4 Results

7.4.1 Ultrastructural changes in cardiac tissue during ischaemia and reperfusion

Adult Sprague-Dawley rat hearts were subjected to left ventricular ischaemia by occlusion of the left anterior descending (LAD) branch of the left coronary artery for up to 1hr, followed by 15 min of reperfusion. After 1hr of ischaemia muscle fibre ultrastructure appeared normal, but some mitochondria were swollen and disrupted. Following 15 min reperfusion the vast majority of the mitochondria were swollen and disrupted compared to those seen in sham-operated hearts as shown below in Figure 60 a-f.

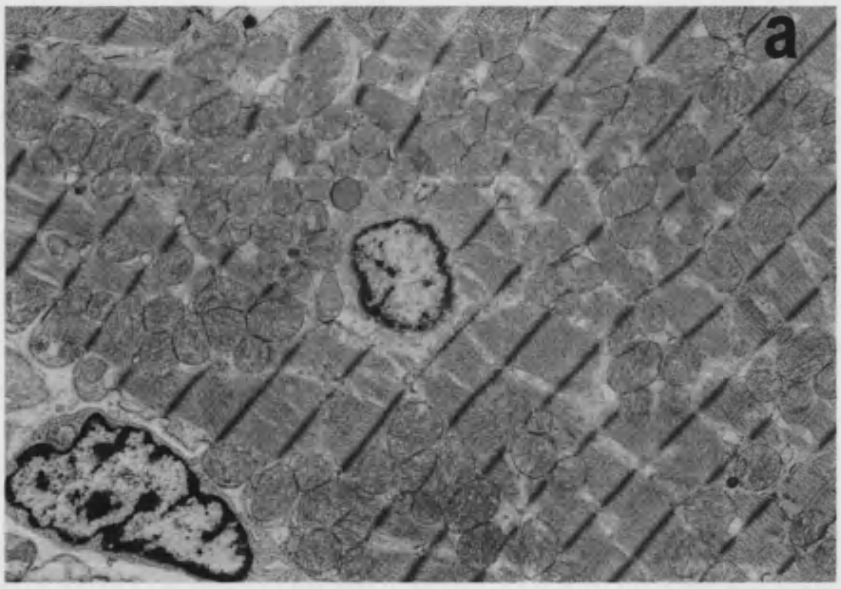
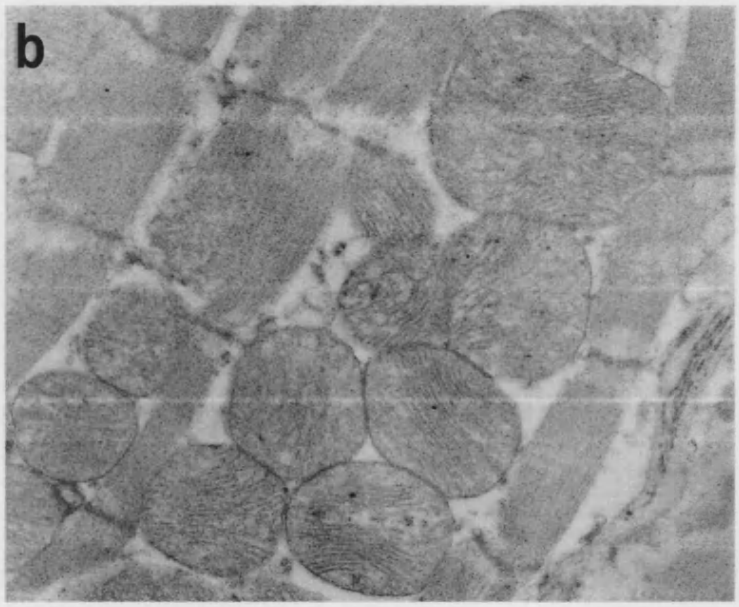
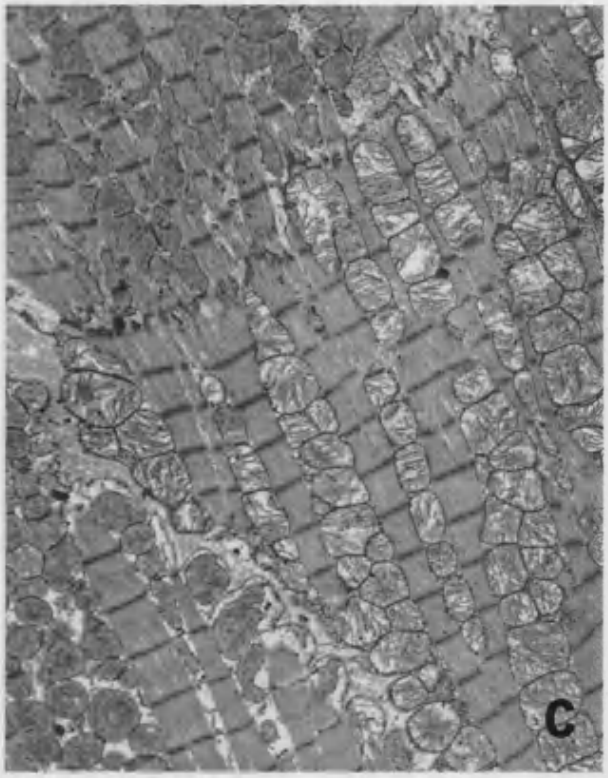


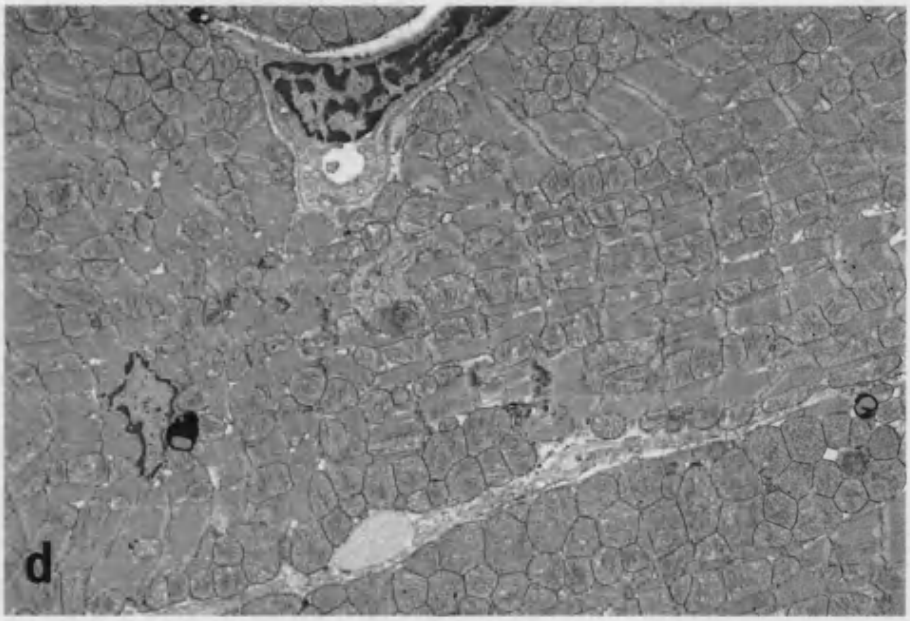
Figure 60a: Electron micrograph illustrating a section of a cardiac myocyte from the left ventricle of a sham-operated heart at total magnification x 6,000. Of interest is the muscle fibre network branching throughout the cytoplasm and the numerous mitochondria with densely packed cristae. A fibroblast may also be observed in the bottom left-hand corner.



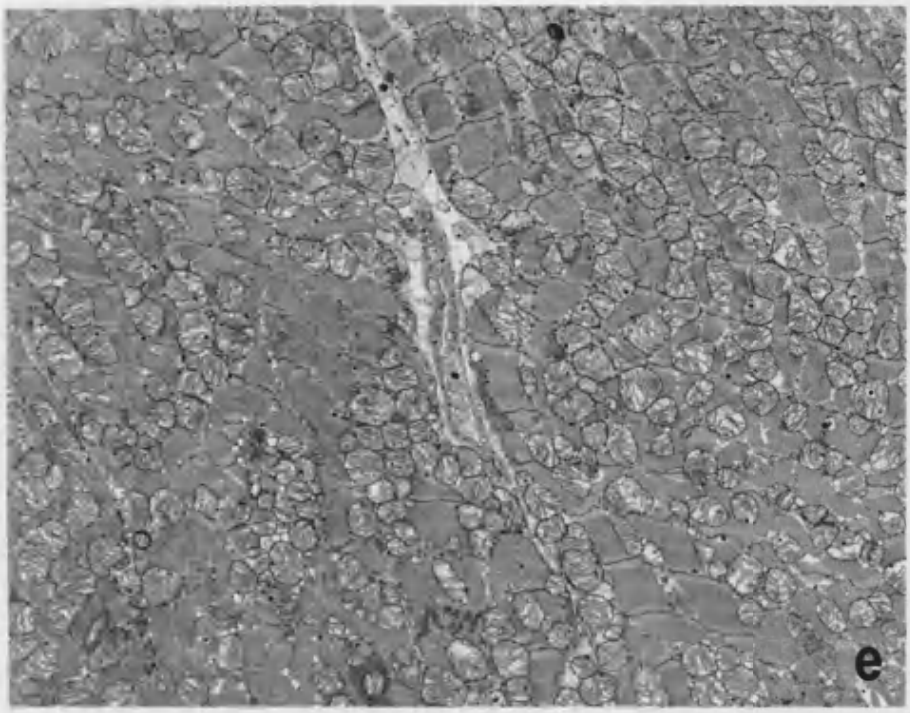
60b: A section from the left ventricle of a sham-operated heart at total magnification x 24,000.

The mitochondria display normal morphology with double membranes and parallel cristae.

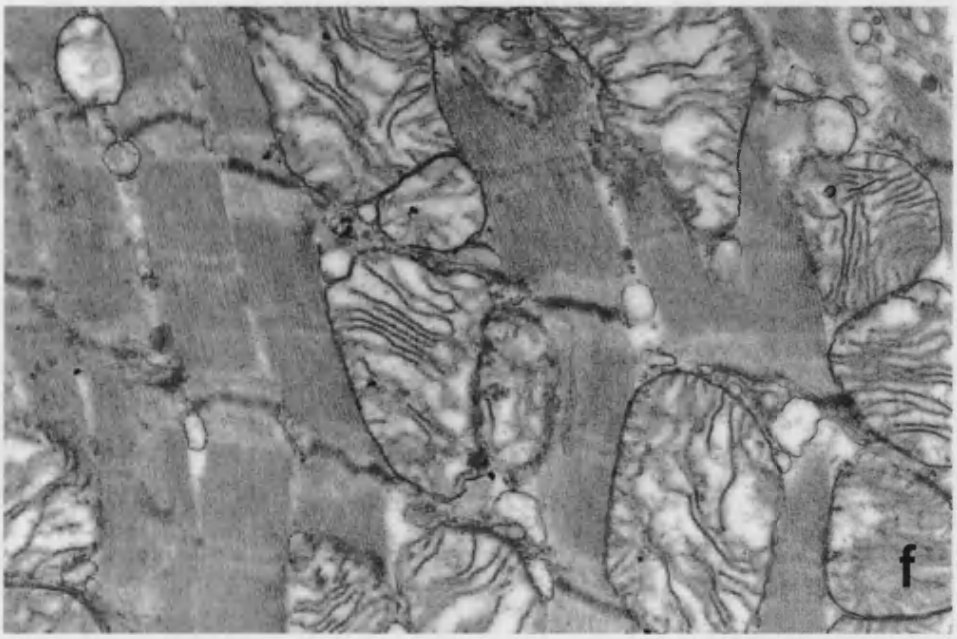




60c & d: Showing sections from the left ventricle of a heart following 60min ischaemia. Normal muscle fibre structure can be observed, but at least 50% of the mitochondria appear swollen and disrupted. (Total magnification x 6,000)



60e: A section from the left ventricle of a heart following 60 min ischaemia and 15 min reperfusion. Normal structure of muscle fibres was consistently seen but almost all of the mitochondria observed were swollen and disrupted. (Total magnification x 6,000)



60f: A section from the left ventricle of a heart following 60min ischaemia and 15 min reperfusion at total magnification x 24,000. This shows swollen mitochondria with disrupted cristae.

7.4.2 Creatine kinase activity in the blood of post-operative rats

LAD occlusion results in damage to the cardiac tissue of the left ventricle as can be seen in Figure 60a-d. This should result in the release of creatine kinase (CK) from the damaged cells that may be observed as an increased level of CK activity in the blood. However as shown below the sham operation, which involved cutting through the intercostal muscles, produced a high background level of CK activity which obscured any small increases caused by the cardiac ischaemia (see Figure 61).

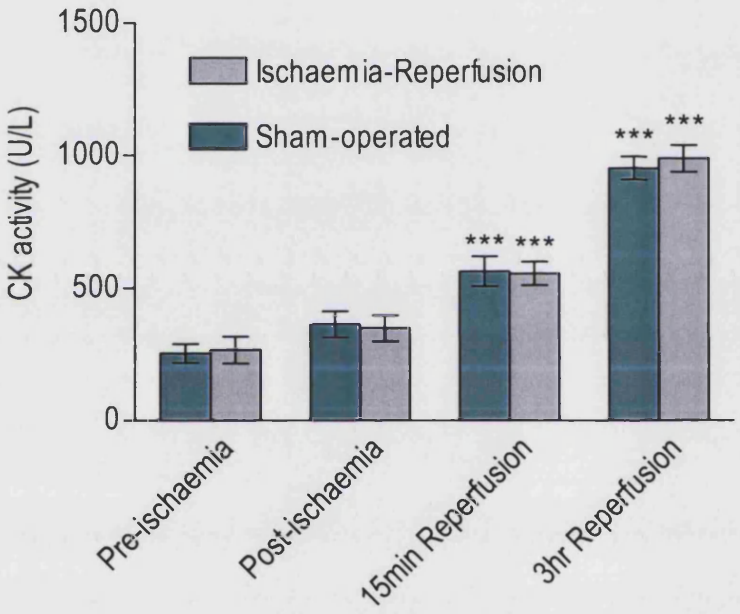


Figure 61: Creatine kinase (CK) activity was measured in the blood of sham-operated rats and those subjected to left ventricular ischaemia and reperfusion. However, the surgical procedure followed in order to expose the heart produced a large cumulative increase in CK activity over the 3-hour period. This obscured any change in CK activity that may have resulted from the LAD occlusion and reperfusion [***, $p < 0.001$ v. pre-ischaemia; n=3 for all groups].

7.4.3 Measurement of mΔΨ in mitochondria isolated from adult rat hearts

The mitochondrial fraction was isolated from 2 sham-operated Sprague-Dawley rat hearts and the ability of the mitochondria to retain tetraphenylphosphonium chloride (TPP^+) was measured using a TPP^+ sensitive electrode. This information was then used to calculate mΔΨ. Upon repeated additions of 5µl of 10mM calcium, a steady decline in mΔΨ was observed culminating in the complete loss of a negative transmembrane charge as shown in Figure 62. The amount of calcium that can be consumed and retained by the mitochondria prior to collapse of mΔΨ is known as the calcium retention capacity (CRC) and is also illustrated below in Figure 62.

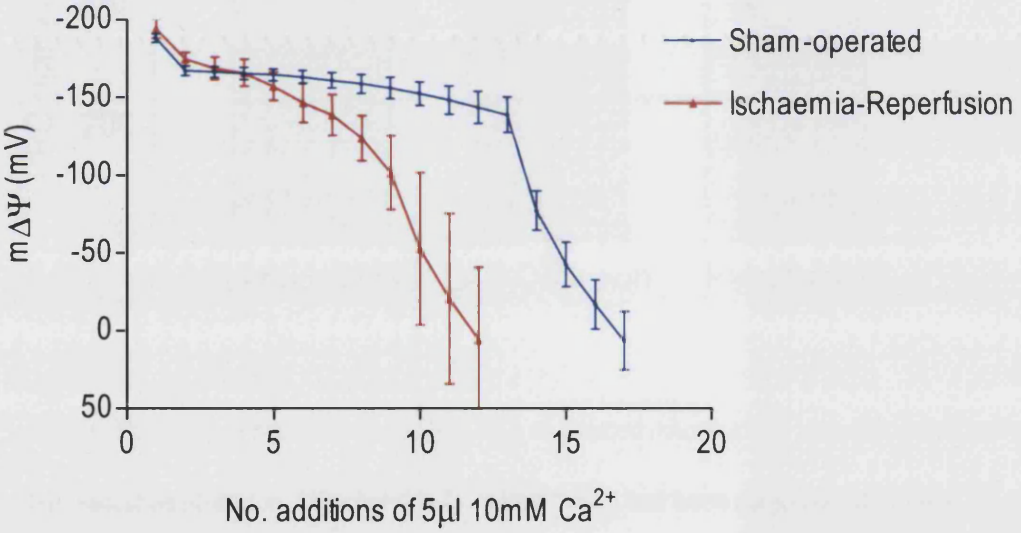


Figure 62: Repeated additions of calcium ions to cardiac mitochondria from sham-operated rats resulted in loss of the mΔΨ until the negative transmembrane charge was completely abolished [n=3 for all groups].

7.4.4 CRC of post-operative and post sham-operative hearts

Mitochondrial fractions were isolated from the left ventricles of 2 rat hearts that had been subjected to either a 1hr sham operation, or 1hr LAD occlusion with or without 15 min reperfusion. $m\Delta\Psi$ of the combined fractions was measured and the calcium retention capacity calculated. Mitochondria from hearts that had been subjected to 15 min reperfusion displayed a lower CRC than those from either the LAD occluded or sham-operated hearts although this result did not attain statistical significance (see Figure 63).

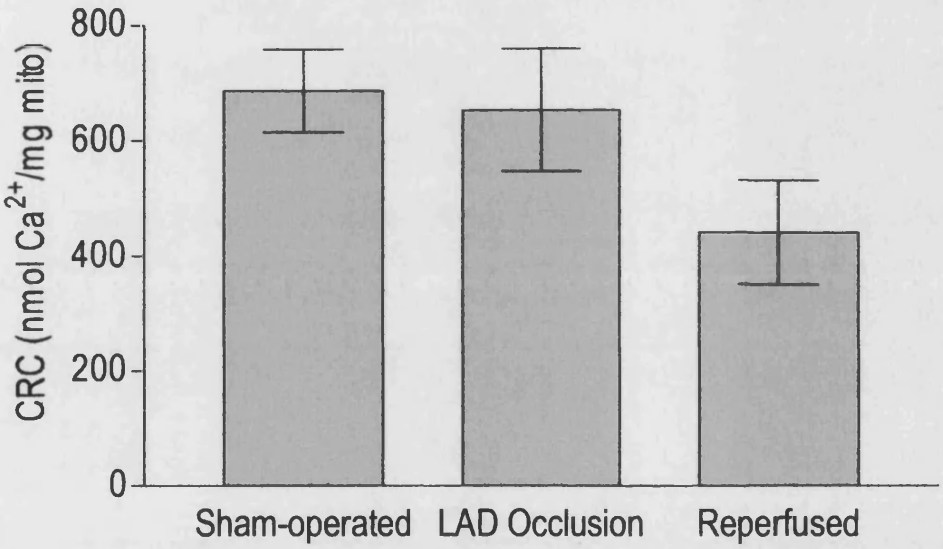


Figure 63: The calcium retention capacity (CRC) of isolated mitochondria was calculated using a TPP^+ sensitive electrode. Mitochondria from hearts that had been subjected to 15 min reperfusion displayed a notable although non-significant reduction in CRC compared to those from either the 1 hr LAD occluded or sham-operated hearts [n=3 for all groups].

7.4.5 PPI-ase activity in the mitochondrial fraction of post-operative rat hearts

The mitochondrial fraction was isolated from post-operative rat hearts and the PPI-ase activity was measured. No variation in the activity of CyPD was observed between the occluded and

reperfused or the sham-operated hearts (Figure 64).

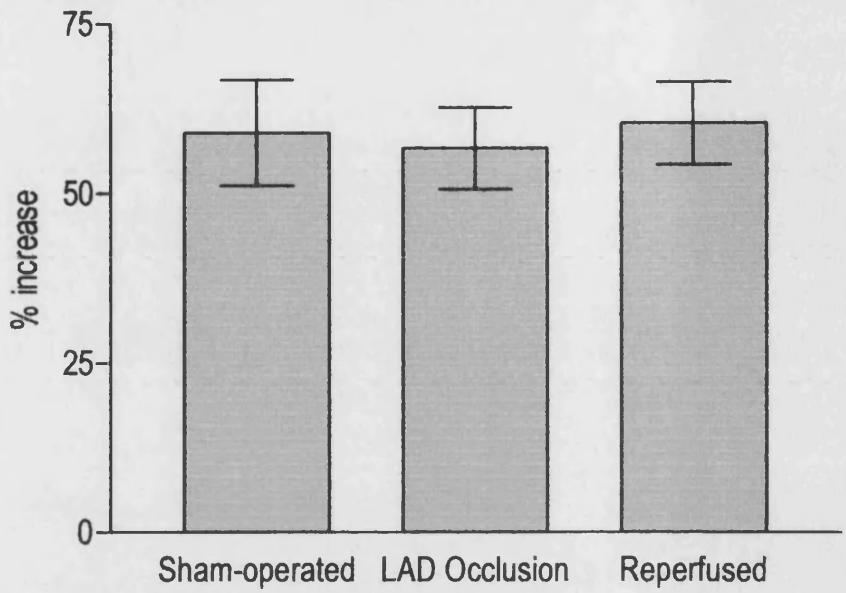


Figure 64: When the PPI-ase activity of the mitochondrial fraction was measured and expressed as the percentage increase in the rate of reaction over an assay with no mitochondria, no variation was seen between the occluded, reperfused and sham-operated hearts [n=3 for all groups].

7.4.6 Identification of Cyclophilin D protein in post-operative rat hearts

The isolated mitochondrial fractions were subjected to Western blotting procedure in order to identify the CyPD protein. No variation in the expression of the CyPD protein was observed between the occluded, reperfused and sham-operated groups (Figure 65).

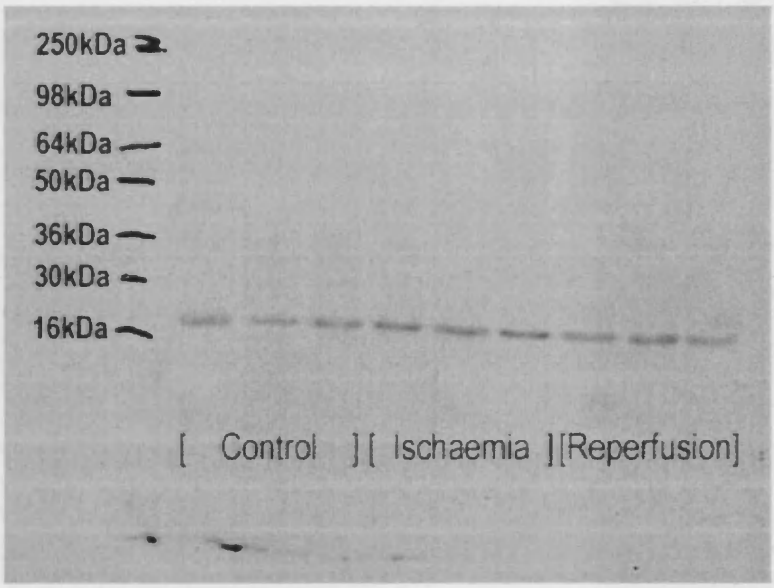


Figure 65: Western blotting of the mitochondrial fraction with an antibody specific to Cyclophilin D (CyPD) revealed protein bands at approximately 18kDa, but no obvious variation in expression between the 1hr LAD occluded, 15min reperfused and sham-operated groups.

7.5 Discussion

Although there have been many convincing studies of the MPTP in isolated cardiomyocytes, the studies performed in intact tissue have been extremely limited. This is clearly due to the problems encountered in measuring the relevant *in vitro* parameters in an *in vivo* model.

The preceding experiments have addressed this issue by attempting to measure the parameters studied in the isolated primary cardiomyocytes in a model of *in vivo* ischaemia-reperfusion injury that has been previously utilised by other researchers.

Morphological analysis by EM was used to gain an overview of tissue damage and to assess mitochondrial swelling and ultrastructure. A quantitative assessment of damage by EM was not performed, as this technique may be considered rather subjective.

Analysis of plasma CK activity is frequently used as a marker of cardiac damage in clinical studies. However, the surgical procedure required to access the heart produced a large increase in plasma CK activity, which obscured any increase caused by the occlusion. The ideal solution would have been to analyse the activity of CK-MB, the cardiac isoform of CK. However, although a standard kit for the detection of human CK-MB is widely available this method proved ineffective for rat CK-MB.

In the primary cardiomyocytes, $m\Delta\Psi$ was measured by flow cytometry using a fluorescent dye that was taken up into energised mitochondria. Ideally, $m\Delta\Psi$ in mitochondria isolated from the reperfused hearts should have been measured using the same dye. However, the extremely small size of the isolated mitochondria was at the limits of detection on the equipment used and accurate readings could not be obtained. Therefore, $m\Delta\Psi$ in isolated mitochondria was calculated according to the ability of the mitochondria to retain TPP^+. This is a method that has been used on numerous occasions by a number of groups (252-257) and has the advantage of being less controversial than $m\Delta\Psi$-sensitive dyes. Measuring mitochondria isolated from the cultured primary cardiomyocytes using the TPP^+ sensitive electrode would have provided a

more direct comparison with reperfused hearts. However, isolating the necessary number of mitochondria for even one electrode measurement from cultured cells proved completely unfeasible. This aptly demonstrates the difficulties inherent in transferring *in vitro* protocols to *in vivo* models.

Analysis of $m\Delta\Psi$ using the TPP^+ sensitive electrode showed a decline in those mitochondria that were isolated from reperfused hearts in comparison to both control and ischaemic hearts. Each treatment group (containing 2 rat hearts) was repeated 3 times and the result was consistent throughout. Although the results obtained were not significant due to a large margin of error, it is anticipated that a larger sample size would produce significant results.

CyPD was analysed in ischaemic and reperfused hearts activity using the same methodology that was employed in the primary cardiomyocytes, allowing a more direct comparison. The PPI-ase activity of the mitochondrial fraction showed no variation between treatment groups and expression of CyPD protein was also unaffected by ischaemia and reperfusion. Thus, the results gained in this model of ischaemia-reperfusion injury are consistent with the results gained in the isolated primary cardiomyocytes.

Ideally the role of CyPD would have been further explored by analysing the effect of CsA on $m\Delta\Psi$ and CyPD activity and expression in reperfused hearts. This could have provided a clearer picture of the functioning of the MPTP *in vivo* and a means of comparison with the results in isolated cardiomyocytes. However, these experiments could unfortunately not be achieved due to pressure of time and resources.

7.6 Conclusions

This preliminary study shows that *in vivo* ischaemia-reperfusion may result in a severe decline in $m\Delta\Psi$ that is not accompanied by any alteration of CyPD activity or expression. These early results are consistent with the data achieved in isolated primary cardiomyocytes but further work is required to provide a clearer picture.

8: Discussion

8.1 Analysis of methodology

8.1.1 Neonatal rat primary cardiomyocytes

Neonatal primary cardiomyocytes were used for all of the *in vitro* experiments performed in this project. These cells were selected as cultured primary cells are generally considered to provide a more accurate reflection of events in whole tissue than a cell line, which accumulates mutations over time. Neonatal cardiomyocytes were used, as obtaining viable cells from neonates is a more straightforward process than using adult rat hearts ensuring more consistent results. Also the implementation of a regular breeding programme ensures that sufficient neonatal myocytes are available on a regular basis. In addition, an original aim of the study was to attempt the transfection of CyPD into primary myocyte cultures, something that is significantly easier to achieve with neonatal cells than with those derived from adults. Unfortunately, unforeseen technical difficulties meant that this was ultimately not attempted. It is worth noting that even in cells derived from the same species, differences in the cellular metabolism can be observed according to the age of animal used, leading to varying experimental observations. For example, a difference in optimal cyclosporin A (CsA) concentration has been noted between neonatal and mature rat cardiomyocytes (133, 136, 237). If significant differences are observed even between different types of primary rat cardiomyocytes, then it is hardly surprising that vastly different results can be seen between different cell lines and primary cells. This is especially true in, for example, the field of apoptosis where the proposed existence of 'mitochondrial' and 'non-mitochondrial' pathways produced a number of conflicting reports and caused much lively discussion. The ideal type of cell to use is often a matter for personal opinion and convenience, but the examples cited above serve to highlight the danger of making generalisations.

8.1.2 Detection of mitochondrial permeability transition pore induction

The detection of mitochondrial permeability transition pore (MPTP) induction in intact cells is a rather problematic area that has attracted much controversy. The most common means of detection rely upon the fact that the opening of the MPTP increases the permeability of the inner mitochondrial membrane thus destroying the mitochondrial transmembrane potential ($m\Delta\Psi$). Various fluorescent dyes have been utilised that are sensitive to $m\Delta\Psi$. Some of the most commonly used are 3,3'dehexylocarbocyanine iodide ($DiOC_6(3)$), tetramethylrhodamine methyl ester (TMRM) and Rhodamine 1,2,3, which are taken up into mitochondria proportionally to the $m\Delta\Psi$. Thus any decrease in fluorescence is proportional to the decline in $m\Delta\Psi$. Another frequently used dye is JC-1 that is incorporated into all mitochondria as a green fluorescent monomer, but only forms red fluorescing aggregates at high $m\Delta\Psi$. All of the above mentioned dyes are widely used, but all have their detractors as well as supporters. ($DiOC_6(3)$), which was utilised in the present study was originally used at 40nM in a variety of intact cells by Zamzami and colleagues (221). Subsequently a study by Mathur and colleagues showed that this protocol was ineffective in cultured cardiomyocytes (244). However, their report failed to take into account the study published by Rottenberg and colleagues that showed that when used at 40nM, ($DiOC_6(3)$) was affected by membrane quenching and they therefore advocated a concentration of less than 1nM (222).

Confocal microscopy results obtained in the primary cardiomyocytes used in this project fully support the work of Rottenberg since 20nM ($DiOC_6(3)$) gave extremely ill-defined staining whereas 0.8nM stained only the mitochondria and diffused into the cytosol upon addition of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (see Chapter 4.4.1).

Where dyes such as those described above can be proved to work, they are extremely

powerful tools for the assessment of $m\Delta\Psi$ in cultured cells. However, they do only measure $m\Delta\Psi$ and therefore provide only an indirect analysis of the MPTP. One notable attempt to directly measure MPTP induction in cells has been made by the group of Lemasters. Isolated hepatocytes were loaded simultaneously with TMRM (tetramethylrhodamine methyl ester) and calcein. In cells with high $m\Delta\Psi$, TMRM localised to the mitochondria whereas calcein stained only the cytosol. Upon induction of the MPTP, the situation was reversed as TMRM was lost from the de-energised mitochondria and calcein staining was observed in both cytosol and mitochondria (258). However, this technique has caused much controversy as Petronilli and colleagues have since published a number of studies disagreeing with the technique (246, 259, 260). The group propose that calcein staining is not observed in the mitochondria only because it is quenched by the presence of TMRM and have put forward an alternative version of the technique. This involves co-staining with calcein and $CoCl_2$ to quench the cytosolic calcein signal so that upon induction of the MPTP $CoCl_2$ enters the mitochondria and the mitochondrial calcein staining is also lost (259). However, Lemasters and colleagues continue to support their original protocol and the issue remains unresolved (145).

During the course of this project, a brief attempt was made to investigate calcein staining. When calcein was loaded into viable, beating primary cardiomyocytes using the protocol of Lemasters, staining of both cytosol and mitochondria was observed. The matter was then not pursued further due to the complexity of the issue. However, if Petronilli and colleagues are correct in their assertion that calcein mitochondrial staining is quenched by TMRM, then the methods of both groups seem rather less straightforward and reliable than the exclusive use of TMRM or a similar dye. Both methods are also further complicated by the proposed physiological opening of the MPTP that could potentially allow the passage of some calcein or $CoCl_2$.

The use of ($DiOC_6(3)$) in this project consistently detected changes in $m\Delta\Psi$, which as

mentioned previously, only implies the involvement of the MPTP. However, the fact that CsA and bongkrekic acid (BA) invariably significantly inhibited the decline in $m\Delta\Psi$ makes it almost certain that at least the majority of the decline was due to induction of the MPTP.

8.1.3 *In vitro* model of ischaemia-reperfusion injury

One of the major areas of difficulty with this study proved to be the model of simulated ischaemia-reperfusion injury, which did not produce the expected behaviour in the cardiomyocytes. The current consensus about ischaemia-reperfusion injury is that while the conditions present in ischaemia are detrimental to the cells, reperfusion can prove to be far more damaging. This is especially true of the initial moments of reperfusion when the MPTP is believed to open. However despite much experimentation with varying periods of simulated ischaemia and reperfusion in this *in vitro* model, results resembling the accepted scenario were never achieved.

The parameters that were relied upon were quantification of cell death and analysis of $m\Delta\Psi$. Cell death was measured according to metabolic conversion of MTT reagent and release of lactate dehydrogenase (LDH) by compromised plasma membranes. Both of these are tried and trusted methods that produced consistent data and there was no reason to suspect that they could have inaccurately reflected the condition of the cells. The measurement of $m\Delta\Psi$ is a rather less certain area. However as discussed above, there is good reason to believe that $m\Delta\Psi$ was quantified with a reasonable degree of accuracy and that the decline in $m\Delta\Psi$ was largely due to the induction of the MPTP.

Assuming therefore that the results described were an accurate reflection of events *in vitro*, the conditions produced by the application of the simulated ischaemia solution followed by simulated reperfusion in culture media must have been at odds with true conditions *in vivo*. Incubation of the cardiomyocytes in the ischaemic solution produced a time-dependent deterioration in the condition of the cells with no accompanying decline in $m\Delta\Psi$. The fact that the number of cells displaying normal $m\Delta\Psi$ remained unchanged after 3 hours of simulated

ischaemia would seem to contradict the cell viability results which showed a distinct deterioration over this period. However, analysis of the fluorescence of the viable cells showed a significant increase in the mean. This would suggest that the least viable cells were dying during the period of simulated ischaemia causing an overall increase in the mean fluorescence. This slight increase could also be due to either CsA or the conditions of the simulated ischaemia such as low pH preventing physiological pore opening and therefore increasing the mean $m\Delta\Psi$. These results correlate both with the cell viability data, which suggest that some cell death was taking place and with the established knowledge that the MPTP does not open during ischaemia. In addition to this, there was also significant suppression of ATP production and very little generation of hydroxyl radicals or hydrogen peroxide (H_2O_2) as expected.

So according to the parameters measured, incubation in the ischaemic solution produced results that were entirely consistent with current knowledge relating to ischaemia.

In contrast, the simulated reperfusion phase produced rather different results. Simulated reperfusion caused only a slight initial decrease in cell viability and no immediate decline of $m\Delta\Psi$ was observed. Induction of the MPTP would be expected to cause rapid cellular death partly due to an inability to generate ATP. Yet a consistent significant decrease in both was only seen after 20 hours of simulated reperfusion and production of ATP recovered significantly and remained surprisingly intact suggesting that the cells were dying gradually over an extended period of time. This could not have been due to the primary cardiomyocytes reaching the end of their natural lifespan since the control cells remained viable and analysis took place on the fourth day after initial plating whereas untreated cardiomyocytes often continued to beat vigorously for 6 days or more. Additionally, whilst application of CsA effectively inhibited the decline of $m\Delta\Psi$, it had no influence over cell death. Taken together,

these results would imply that the MPTP did not play a significant role in this model of ischaemia-reperfusion injury and opened only as a consequence of the death of the primary cardiomyocytes.

Therefore, it could reasonably be concluded that this model did not accurately replicate the conditions present during *in vivo* ischaemia-reperfusion injury that lead to induction of the MPTP. At present, the conditions that lead to opening of the MPTP are believed to include high matrix Ca^{2+} concentration, accumulation of free radicals, depleted ATP and accumulation of inorganic phosphates. In addition the inhibitory effect of the low pH present during ischaemia is removed, further encouraging MPTP induction.

The solution developed by Esumi and colleagues is designed to simulate the extracellular environment of myocardial ischaemia (217). It was extremely successful in the severe suppression of ATP production during simulated ischaemia, which also strongly implies the presence of an excess of inorganic phosphates in the mitochondria. These unfortunately could not be accurately measured due to the huge number of cardiomyocyte mitochondria required to gain an accurate reading in a standard assay. Incubation of primary cardiomyocytes in the ischaemic solution also resulted in significant generation of free radicals.

The only conditions that remain entirely unknown are the Ca^{2+} concentration in the mitochondrial matrix, and the intracellular pH. These parameters are dependent upon the Na^+/Ca^{2+} antiporter and the Ca^{2+} ATPase, and the Na^+/H^+ antiporter respectively. It is therefore possible that a component of either the ischaemia solution or the maintenance media interfered with the normal functioning of one of these. Alternatively the Na^+ concentration of the ischaemic solution may have been incorrect, especially in view of the fact that it incorporated sodium dithionite in place of the KCN of the original solution.

The pattern of cell injury over time does provide a clue to the nature of the injury. When cardiomyocytes were subjected to only1hr of treatment with the ischaemic solution, metabolic

activity as measured by the conversion of MTT reagent did not show a significant decrease. However when the cells were subsequently incubated in full media for 24hr, the same pattern of cell death was observed as occurred following longer periods of simulated ischaemia. This could imply that elements of the ischaemic solution remained inside the cells causing a gradual and irreversible loss of viability, or were metabolised into a more damaging form. As the majority of the solution is based on Krebs buffer and consists purely of salts, the most likely candidates are the 2-deoxyglucose, the sodium lactate and the sodium dithionite.

Slight allowances must be made for the fact that these experiments were performed on neonatal primary cardiomyocytes whereas Esumi and colleagues were studying cardiomyocytes isolated from adult rats. As mentioned previously, the age of the animals used can have a major influence on the results obtained.

A number of other *in vitro* simulations of ischaemia have been inflicted upon isolated cardiomyocytes with a varying range of effects (133, 239, 241). Ideally some of these other simulations would have been applied to the neonatal primary cardiomyocytes. Unfortunately however, this was not feasible within the scale of this project.

Finally, it must be stressed that no *in vitro* simulation, regardless of how well researched can hope to be completely accurate. Although these simulations are very useful research tools that can substantially reduce the amount of necessary animal experimentation, they can never completely substitute for *in vivo* protocols.

8.1.4 Use of staurosporine as an inducer of apoptosis

During the period in which the model of apoptosis in primary cardiomyocytes was set up, chemical induction was the most common method of initiating apoptosis. Frequently published examples included chemotherapeutic agents such as daunomycin and doxorubicin (36, 261-263), nitric oxide (264, 265) and staurosporine (SSP) (35, 39, 266). Two other methods of induction that were also employed were oxidative stress (29, 267) and Fas ligand (14, 268, 269). Of these, oxidative stress was an inappropriate method for a study in which free radical activity was to be measured, but induction of apoptosis using anti-Fas antibody was briefly attempted. Although it reputedly induced the 'mitochondrial' pathway of apoptosis signalling, anti-Fas antibody application had no effect on the neonatal primary cardiomyocytes (data not shown). In fact, several recent publications seem to confirm that cardiac myocytes are resistant to Fas induced apoptosis and require a co-stimulant (270-272).

Of the chemical inducers of apoptosis that were available and appeared to successfully induce apoptosis in primary cardiomyocytes, SSP was selected for the following reasons.

Firstly, SSP was considered to induce 'true' apoptosis as it produced the generally accepted morphological features (43, 273), with these changes occurring in a sequence typical for apoptosis (274). Additionally, the process usually required the involvement of the Ced-3/ICE family of proteins, now commonly known as caspases and considered to be one of the main hallmarks of apoptosis (39, 275). A second advantage of the use of SSP was the fact that it was known to induce apoptosis in a number of cell types without the need for any protein synthesis (233), showing that it acted through a pathway involving proteins that were constitutively expressed in most types of cell. SSP is a broad-spectrum inhibitor of protein kinases and it is generally assumed that this inhibitory affect that gives SSP its ability to induce apoptosis. However, no study has as yet demonstrated this and it may not necessarily be the case as a detailed study of another protein kinase inhibitor, chelerythrine, showed that it

induced apoptosis in primary cardiomyocytes through the generation of reactive oxygen species (276). Although some anucleate cells appear resistant to SSP induced apoptosis, experiments in cell free systems have shown that a component in the cytoplasm is essential for the induction of DNA fragmentation by SSP (43). This demonstrates that SSP could act upon a number of intracellular proteins in order to induce apoptosis. Finally, SSP had already been successfully utilised for the induction of apoptosis in primary cardiomyocytes (35, 266) including those derived from neonatal rats (39) and the combination of these points made it a seemingly reliable option.

In more recent papers, serum deprivation appears to be the most frequently published method of apoptosis induction in a wide variety of cells (267, 277-279) including cardiomyocytes (280, 281). However, as nutrient deprivation is also a feature of ischaemia (40) it was not considered suitable for this project, where three separate and distinct models of cell death were sought. Currently, the accumulated evidence would suggest that apoptosis during ischaemia-reperfusion injury occurs mainly on the fringes of the affected area and its most important role may be during the remodelling that can transpire following the event. Whether apoptosis occurs via the 'death receptor' or the 'mitochondrial' pathway in these situations is as yet unknown.

8.2 Overview and hypothesis

Cardiac disease, especially myocardial infarction, is one of the leading causes of human mortality in Western society. Ischaemia-reperfusion injury, which can result from myocardial infarction, is an important and extremely complex type of injury in solid tissues. Necrosis and potentially also apoptosis have an important role in ischaemia-reperfusion injury and induction of the MPTP has been observed during reperfusion as well as cited as a major factor in both forms of cell death.

Various aspects of the MPTP have been studied in many different *in vitro* models of cell death and, sparsely, in isolated perfused organs. This project aimed to bring together varying strands of research in the context of isolated primary cardiomyocytes, which arguably offer a more genuine insight than established cell lines. An additional aim was to attempt to reproduce some of the protocols in an *in vivo* model of ischaemia-reperfusion injury.

The focus of the project was the molecular chaperone protein cyclophilin D (CyPD) that has frequently been implicated in induction of the MPTP. The purpose of the project was to examine the importance of CyPD to MPTP induction in a variety of situations and to study the implications.

8.3 General Discussion

Three models of cell death were set up in neonatal primary cardiomyocytes. Necrotic cell death was caused by both application of t-butyl hydroperoxide (t-BOOH) and subjection to simulated ischaemia-reperfusion injury and was measured by conventional, frequently used assays. Measurement of apoptotic cell death (induced using the established initiator SSP) is a more controversial area and was therefore analysed by a range of methods designed to assess different aspects of apoptosis. As pathological apoptosis can manifest itself in various forms, presumably according to the amount of intracellular energy available, measuring only one parameter can potentially produce unreliable results. Therefore the multi-stranded approach utilised was considered more suitable than a single method such as, for example, staining the condensed chromatin of apoptotic cells with Hoechst 33342.

In order to fully study the role of the MPTP and CyPD in cell death, the effects of 2 different inhibitors of the MPTP were studied. BA is a specific inhibitor of the adenine nucleotide translocase (ANT) that is not known to affect CyPD and was used as an aid to establishing the importance of the MPTP in each model. CsA is a well-characterised inhibitor of CyPD that was used to help establish both the importance of the MPTP and the role of CyPD in cardiomyocyte cell death. BA proved to give variable results and adversely affected cell viability in the models of necrosis and simulated ischaemia-reperfusion injury so reliable results were only obtained for apoptosis. It must be noted at this point that BA binding to ANT inevitably effects the movement of adenosine nucleotides in the cell. However, at the concentrations used it had no detectable influence on cellular metabolism in control cardiomyocytes. CsA showed no adverse affect on viability but was only able to inhibit t-BOOH induced necrotic cell death. An obvious explanation for this would be that the MPTP was induced only in the model of necrotic cell death. However when $m\Delta\Psi$ was measured, all models showed a marked decrease, which

moreover was significantly inhibited by CsA. BA also successfully inhibited the decline in $m\Delta\Psi$ when tested in the model of apoptosis. This then would suggest either that blocking induction of the MPTP had an inhibitory effect on cell viability that was short-lived and easily overcome, or that induction of the MPTP was not a crucial event in these processes of cell death. In the case of apoptosis, loss of DNA fragments was seen earlier in the process than any decrease in $m\Delta\Psi$. As DNA fragmentation is known to be one of the endpoints of apoptosis, it follows that induction of MPTP and the corresponding loss of $m\Delta\Psi$ is likely to be a consequence of cell death as opposed to a part of the active process of SSP-induced apoptosis. This is also supported by the fact that $m\Delta\Psi$ in the viable cell population remained high throughout the experiment. Therefore, application of CsA or any other inhibitor of the MPTP would be unable to aid cell survival. It must be stressed that many different versions of the process of apoptosis have been published and the relative importance of the MPTP may vary according to the inducer of apoptosis and the cell type. However, this does effectively demonstrate the complexity of the process and the problems inherent in finding an effective method of inhibition that would cover a wide range of situations.

In the model of necrotic cell death, CsA successfully inhibited both the decline in $m\Delta\Psi$ and the onset of cell death. The fact that CsA inhibition of cell death was only very partial is probably due to the extreme severity of the insult, suggesting that other factors beside MPTP induction were contributing to cell death. These results show that induction of the MPTP is an important event in this model of rapid necrosis due to severe oxidative stress and that CyPD may play an important role.

Cardiomyocyte death due to simulated ischaemia-reperfusion injury was completely unaffected by CsA application despite the fact that significant inhibition of the MPTP was observed. This result is contrary to other published studies including the results obtained by Griffiths and

colleagues in isolated, perfused hearts (136) and may therefore suggest that the cellular model used in this project is not altogether accurate. This supposition is supported by the fact that $m\Delta\Psi$ remained high for several hours following simulated reperfusion whereas other groups have shown that induction of the MPTP occurs almost immediately upon reperfusion.

The majority of the direct evidence in the scientific literature for the importance of MPTP induction is provided by studies of inhibitors such as CsA and BA. This data must be treated with a certain amount of caution since these chemicals are known to have other effects in the cell. CsA is extremely toxic at higher concentrations and has been shown to induce apoptosis in a variety of cell types (212-214, 235). It also has a multitude of other functions such as stimulation of tumour growth factor (TGF)-β production (282) and inhibition of the other members of the CyP family resulting in the well-characterised suppression of the immune response via calcineurin binding. In cardiac cells, this pathway is known to influence the cellular growth response. Although the potential role of CsA in this response is controversial and unclear, there is a distinct possibility that its application to cardiac myocytes could influence cell death, especially apoptosis, via interaction with calcineurin. In this study, inhibition of the MPTP was confirmed by confocal microscopy but other effects in the cell were not completely accounted for. Although CsA was not detectably toxic in the control cardiomyocytes, there is a possibility that its presence could have exacerbated cell death in any of the models used. One possible means of testing this could be the application of FK506, which is known to inhibit calcineurin by binding to FK-binding proteins, without affecting the MPTP. As it acts on the calcineurin pathway in much the same way as CsA it should, in theory, replicate any influence of CsA on cell death that is unrelated to the MPTP.

BA is less well studied but, in particular, allowances must be made for its effect on cell

metabolism through its ability to inhibit the activity of the ANT. The data in this project strongly suggests that it could have an uncertain effect on cell viability in some situations. As BA was used in extremely small volumes (less than 5µl/ml) at concentrations that were shown to be completely non-toxic in control primary cardiomyocytes and did not appear to inhibit metabolic activity, it is presumed that BA was either influencing some other aspect of necrosis or attaining toxicity through interaction with t-BOOH and the ischaemic solution. It cannot then be stated with absolute confidence that the beneficial action of these inhibitors is due entirely to their effect on the MPTP. However since 2 such different MPTP inhibiting chemicals as CsA and BA can reduce cell death in such a wide variety of situations as are to be found in the literature as well as in this study, the importance of the MPTP is a near certainty.

In all the situations studied in this project CsA inhibition of $m\Delta\Psi$ was consistently observed, strongly implicating the involvement of CyPD. Analysis of CyPD activity and expression showed that both remained unaffected by cell death. However application of CsA resulted in a significant inhibition of CyPD peptidyl prolyl *cis-trans*-isomerase (PPI-ase) activity in correlation with CsA inhibition of $m\Delta\Psi$. This would imply that the site of PPI-ase activity in CyPD is important for binding to the MPTP and influencing its induction despite that fact the onset of cell death did not result in any change in activity. The PPI-ase activity of the cyclophilin family has been shown to be essential for protein folding and it has been suggested that CyPD may induce a conformational change in ANT that sensitises it to the action of Ca^{2+} thus allowing induction of the MPTP (157). It is quite possible that no increase in PPI-ase activity is necessary for the implementation of this scenario or that the change in activity is too small or short lived to be detectable by this particular assay. Alternatively, it may be that the effect of CsA binding to the CyPD PPI-ase active site is to either inhibit its binding to ANT or to create a physical barrier obstructing the ANT Ca^{2+} binding site and PPI-ase activity is not necessary for

MPTP induction.

In all models of cell death studied, it was found that CsA was binding to CyPD and resulting in inhibition of the MPTP. Despite this no inhibition of apoptosis or simulated ischaemia-reperfusion injury could be seen, and only a slight inhibition of necrosis was observed.

However, cell death is an extreme endpoint and therefore a rather crude indicator, so it was undertaken to measure other vital parameters that would precede and possibly be exclusive of, cell death. These experiments were also designed to provide an insight into the events surrounding MPTP induction in these models of cell death.

CsA proved unable to influence the generation of free radicals noted during necrosis and simulated ischaemia-reperfusion injury. These results are consistent with the theory that generation of free radicals in cardiomyocytes is initiated during ischaemia and will therefore remain unaffected by inhibition of MPTP induction during reperfusion. The fact that significant generation of free radicals was observed shows that there are potentially many important parameters of ischaemia-reperfusion injury that are independent of MPTP induction and therefore cannot be influenced by MPTP inhibition.

ATP production was significantly reduced during apoptosis and necrosis, but CsA was only able to affect the reduction observed during necrosis. As a high $m\Delta\Psi$ is known to be crucial for the production of ATP, these results are consistent with the probability that MPTP induction is an important element of t-BOOH initiated necrosis but only a consequence of SPP-induced apoptosis. ATP production was severely depressed during simulated ischaemia, but after 20hr of simulated reperfusion it had significantly recovered. Neither result was significantly affected by the application of CsA. Suppression of ATP production during simulated ischaemia was

expected but such significant recovery after 20hr of simulated reperfusion is inconsistent with the drop in $m\Delta\Psi$. This again suggests that MPTP was a late consequence of cell death and is most likely to be due to inaccuracies in the *in vitro* model of ischaemia-reperfusion injury as previously discussed.

In this project complete inhibition of the MPTP by either CsA or BA was never observed. It may be that the concentrations required for complete inhibition were toxic to the cardiomyocytes, or it may imply that MPTP induction was occurring despite the presence of the inhibitors. The precise manner by which the MPTP is formed is unknown as ANT individually displays pore-like properties and other components of the pore remain disputed. If CsA acts to prevent CyPD binding, then the drop in $m\Delta\Psi$ that was independent of CsA or BA inhibition could have been due to the action of ANT alone. If, as has been proposed CyPD interacts physiologically with ANT (160, 161), then logically not all CyPD binding could be prevented by CsA meaning some permeability will inevitably result. Alternatively, if CsA can interact with CyPD that is already bound to ANT providing mainly a physical block as has also been suggested (159), then this could also account for the partial inhibition observed.
It should be noted that, had complete inhibition of the MPTP been attained, this could have influenced other parameters, most notably the decline in ATP production where a positive inhibition could possibly have resulted.

The inability of CsA to attenuate to injury to primary cardiomyocytes during simulated reperfusion following simulated ischaemia disagrees with previous findings including those of Griffiths and colleagues in isolated perfused hearts (136). As previously stated, this is most likely to be due to failings of the *in vitro* model of ischaemia-reperfusion injury. However, even results obtained from isolated, perfused hearts cannot be as reliable as an equivalent study *in*

vivo due to the extra stresses placed on a heart during isolation and the possibility of a lacking vital ingredient contained in circulating blood or surrounding tissue.

For that reason, the $m\Delta\Psi$ and CyPD expression and activity were analysed in rat hearts subjected to ischaemia-reperfusion injury whilst remaining *in situ*. The lack of any detectable change in CyPD activity or expression was consistent with the results obtained in isolated primary cardiomyocytes. However, analysis of $m\Delta\Psi$ showed a striking albeit non-significant decline in mitochondria isolated from reperfused hearts. Even in this situation $m\Delta\Psi$ measurements are undoubtedly influenced by the removal and isolation of the mitochondria. However, any deterioration will be consistent between the treatment groups and this method provides the means to keep the heart in its natural setting during the ischaemia and reperfusion. These initial measurements of $m\Delta\Psi$ although inconclusive broadly correlate both with the data obtained from the primary cardiomyocytes and with the published studies of other groups. As measurement of $m\Delta\Psi$ is acceptable by many as a means of studying MPTP induction, this method is well worth pursuing. In particular, analysing the effect of CsA could, firstly provide additional evidence that any observable decrease in $m\Delta\Psi$ is caused by the opening of the MPTP and secondly, give further information concerning the importance of CyPD in ischaemia-reperfusion injury.

In addition to this, the influence of CsA on parameters of cardiac function such as electrical activity and left ventricular blood pressure could be measured by standard techniques. Along with analysis of the ATP content of isolated mitochondria by a modification of the luciferase assay used in this project, or tissue ATP content by HPLC (high performance liquid chromatography) procedures, this would give a more complete picture of the functioning of the MPTP during ischaemia-reperfusion injury and the role of CyPD.

8.4 Conclusions and future directions

Pathological induction of the MPTP has attracted a great deal of attention and funding over recent years. It is important to note that physiological induction of the MPTP has been demonstrated (127) but the cumulative evidence strongly suggests that induction of the MPTP is almost certainly an important pathological event.

Although many groups have pointed to evidence of apoptosis during ischaemia-reperfusion injury, necrotic cell death is still regarded as the predominant feature. The potential importance of the MPTP in reperfusion provoked necrosis is evidenced by the observation that mitochondrial swelling is one of the earliest changes seen in necrosis including that caused by reperfusion, whereas in apoptosis mitochondrial morphology remains unchanged (283).

As evidenced in this project, the MPTP may often be induced as a consequence of irreversible cell death. This highlights the fact that inhibition of the MPTP may not necessarily be beneficial even if induction is observed. Even where MPTP induction is an important occurrence there are ischaemic events such as generation of free radicals outside of the influence of the MPTP that could ultimately result in the death of the cell.

Although the influence of the MPTP was variable, the results of this project showed that CsA significantly inhibited induction of the MPTP in a wide variety of circumstances. This strongly supports the hypothesis that CyPD plays a vital role in the formation of the MPTP and is therefore of great significance in any situation where the MPTP is pathologically induced. It is thought that given severe enough conditions, especially in a situation of extremely high Ca^{2+}, the MPTP will be induced irrespective of the action of CyPD (187-189). However under such circumstances, it is likely that irreversible cell death would occur regardless of MPTP induction.

Ischaemia-reperfusion injury is a large and burgeoning field with a huge number of different focuses of study. Besides induction of the MPTP, numerous other intracellular events occur during ischaemia-reperfusion injury including activation of MAPK pathways (284), generation of free radicals (109, 249, 285) and movement of inorganic ions to mention but a few. The fact that injury has been attenuated by inhibitors as varied as Mg^{2+} (286), pyruvate (287), caspase inhibitors (38), the vitamin E analogue Trolox (288), the phytoestrogen genistein (289) and the gap junction uncoupler heptanol (290) demonstrates the huge complexity of interacting pathways occurring in ischaemic and reperfused cells. Some of these such as the generation of free radicals can in fact contribute to MPTP induction, an occurrence that is exacerbated by the inhibition of free radical scavengers such as SOD and catalase during ischaemia. This highlights the fact that there may be many effective methods of protecting patients from ischaemia-reperfusion injury. The remedies that eventually come into use may be determined more by ease of use, expense and marketing rather than purely by scientific and clinical merit. However, ischaemia-reperfusion injury occurs with varying severity according to factors such as the length of the ischaemic period and the general health of the affected tissue. In many instances the damage is irreversible regardless of drug interventions, in other cases rapid and appropriate treatment could save the life of a patient. In a situation of life-threatening cardiac ischaemia-reperfusion injury, inhibition of the MPTP is arguably the most important factor to attempt to control since without adequate production of ATP the cell would almost certainly die even if many other detrimental events were successfully contained.

In this scenario, the inhibition of CyPD has a distinct advantage. CsA is an extremely well characterised drug that is already widely used for immuno-suppression so the toxicity and side effects are well known. In the context of cardiac ischaemia-reperfusion injury, its influence could be tested during for example, bypass operations by measuring CK-MB in the blood of control and treated groups. This would be extremely straightforward, non-invasive and

inexpensive and could eventually greatly ease one of the major dangers associated with heart surgery. In the case of myocardial infarctions, the majority of these are precipitated by thrombosis and patients are treated with fibrinolytic drugs to allow reperfusion to the affected area. CsA could be administered in combination with these drugs to prevent the reperfusion injury that can be potentially more serious than the infarction itself. In addition, there are analogues of CsA such as *N*-methylVal-4-cyclosporin that bind CyPD and inhibit the MPTP without also causing immuno-suppression. In a patient undergoing and recovering from major surgery, this would be an important consideration. Although such analogues are not yet as well characterised as CsA, they may have potential for the attenuation of ischaemia-reperfusion injury without some of the side effects associated with CsA.

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